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EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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CONTENTS.

No. 1, JANUARY 1, 1924.

PAGE

COHN, ALFRED E., and SWIFT, HOMER F. Electrocardiographic evidence of myocardial involvement in rheumatic fever. Plates 1 to 3..... 1

DU NOÜY, P. LECOMTE. Surface tension of serum. VII. Significance of the maximum time-drop of serum solutions..... 37

DETWEILER, HERBERT K., and HODGE, W. RAY. An attempt to isolate a filter-passing virus in epidemic influenza..... 43

MCCARTNEY, JAMES E. Brain lesions of the domestic rabbit. Plates 4 to 8..... 51

NICHOLSON, F. M. An experimental study of mitochondrial changes in the thyroid gland. Plates 9 to 11..... 63

ROUS, PEYTON, MCMASTER, PHILIP D., and DRURY, DOUGLAS R. Observations on some causes of gall stone formation. I. Experimental cholelithiasis in the absence of stasis, infection, and gall bladder influences. Plates 12 to 15..... 77

ROUS, PEYTON, DRURY, DOUGLAS R., and MCMASTER, PHILIP D. Observations on some causes of gall stone formation. II. On certain special nuclei of deposition in experimental cholelithiasis. Plates 16 to 19..... 97

INGVALDSEN, THORSTEN, WHIPPLE, ALLEN O., BAUMAN, LOUIS, and SMITH, BEVERLY C. The rôle of anhydremia and the nature of the toxin in intestinal obstruction..... 117

WEBSTER, LESLIE T. Microbic virulence and host susceptibility in paratyphoid-enteritidis infection of white mice. III. The immunity of a surviving population..... 129

FELTON, LLOYD D., and DOUGHERTY, KATHARINE M. Studies on virulence. II. The increase in virulence *in vitro* of a strain of pneumococcus..... 137

FELTON, LLOYD D., and DOUGHERTY, KATHARINE M. Studies on virulence. III. Influence of hydrogen ion concentration and of ingredients of plain broth on the virulence of pneumococci..... 155

TALIAFERRO, WILLIAM H. A reaction product in infections with *Trypanosoma lewisi* which inhibits the reproduction of the trypanosomes..... 171

No. 2, FEBRUARY 1, 1924.

FLEXNER, SIMON, and AMOSS, HAROLD L. Revived activity of the virus of poliomyelitis..... 191

ROBERTSON, OSWALD H., SIA, RICHARD H. P., and WOO, SHU-TAI T. Studies on pneumococcus growth inhibition. I. The protective action of gelatin for pneumococci in suspension..... 199

ROBERTSON, OSWALD H., and SIA, RICHARD H. P. Studies on pneumococcus growth inhibition. II. A method for demonstrating the growth-inhibitory and bactericidal action of normal serum-leucocyte mixtures. Plate 20..... 219

SHIBLEY, GERALD S. Studies in agglutination. I. The agglutination of streptococci..... 245

PRITCHETT, IDA W. Homologous and heterologous protection in mice vaccinated with the two types of mouse typhoid bacillus..... 265

AVERY, OSWALD T., and MORGAN, HUGH J. The occurrence of peroxide in cultures of pneumococcus..... 275

AVERY, OSWALD T., and MORGAN, HUGH J. Studies on bacterial nutrition. V. The effect of plant tissue upon the growth of anaerobic bacilli..... 289

SMITH, THEOBALD, and LITTLE, RALPH B. Proteinuria in new-born calves following the feeding of colostrum..... 303

HOWE, PAUL E. The relation between the ingestion of colostrum or blood serum and the appearance of globulin and albumin in the blood and urine of the new-born calf..... 313

HADEN, RUSSELL L., and ORR, THOMAS G. The effect of inorganic salts on the chemical changes in the blood of the dog after obstruction of the duodenum..... 321

No. 3, MARCH 1, 1924.

OLITSKY, PETER K., and MCCARTNEY, JAMES E. Experimental studies on the etiology of typhus fever. VI. Skin lesions in experimental typhus fever of guinea pigs..... 331

MORGAN, HUGH J., and AVERY, OSWALD T. Growth-inhibitory substances in pneumococcus cultures..... 335
AVERY, OSWALD T., and NEILL, JAMES M. Studies on oxidation and reduction by pneumococcus. I. Production of peroxide by anaerobic cultures of pneumococcus on exposure to air under conditions not permitting active growth..... 347
AVERY, OSWALD T., and NEILL, JAMES M. Studies on oxidation and reduction by pneumococcus. II. The production of peroxide by sterile extracts of pneumococcus..... 357
BLOOMFIELD, ARTHUR L., and FELTY, AUGUSTUS R. Quantitative factors in "test-tube infection."..... 367
LEAKE, CHAUNCEY D., VICKERS, J. L., and BROWN, THOMAS K. Observations on blood reaction in experimental pneumonia..... 393
DRURY, DOUGLAS R., MCMASTER, PHILIP D., and ROUS, PEYTON. Observations on some causes of gall stone formation. III. The relation of the reaction of the bile to experimental cholelithiasis..... 403
ROUS, PEYTON, and MCMASTER, PHILIP D. The liver requirement of the fasting organism. Plate 21..... 425
STRONG, LEONELL C. Indications of tissue specificity in a transplantable sarcoma..... 447
SCOTT, W. J. M. The influence of the adrenal glands on resistance. II. The toxic effect of killed bacteria in adrenalectomized rats..... 457
LEE, FERDINAND C. The effect of pilocarpine on the number of small lymphocytes in the circulating blood following ligation of the thoracic duct..... 473
LYNCH, CLARA J. Studies on the relation between tumor susceptibility and heredity. I..... 481

No. 4, April 1, 1924.

SWIFT, HOMER F. The pathogenesis of rheumatic fever. Plates 22 to 27..... 497
BRONFENBRENNER, JACQUES J., and SCHLESINGER, MONROE J. The effect of digestive juices on the potency of *botulinus* toxin..... 509

BRONFENBRENNER, JACQUES J., and WEISS, HARRY. The effect of anesthesia and of sedatives on the serum therapy of experimental botulism..... 517
MCCARTNEY, JAMES E. Experiments on the survival of the febrile herpetic and allied viruses *in vitro*..... 533
AVERY, OSWALD T., and NEILL, JAMES M. Studies on oxidation and reduction by pneumococcus. III. Reduction of methylene blue by sterile extracts of pneumococcus..... 543
CHESNEY, ALAN M., and KEMP, JAROLD E. Experimental observations on the "cure" of syphilis in the rabbit with arsphenamine..... 553
MORGAN, HUGH J. The correlation of certain phenomena occurring during the growth of pneumococcus..... 565
FISCHER, ALBERT. The interaction of two fragments of pulsating heart tissue. Plate 28..... 577
FISCHER, ALBERT. The differentiation and keratinization of epithelium *in vitro*. Plate 29..... 585
MOOSER, H. Experimental studies with a spiral organism found in a wild rat. Plates 30 to 32..... 589
BROWNLEE, JOHN. The rate of healing of wounds..... 603
BURKE, VICTOR, and SKINNER, CHARLES E. The reverse selective bacteriostatic action of acid fuchsin..... 613

No. 5, MAY 1, 1924.

FLEXNER, SIMON, and AMOSS, HAROLD L. An immunizing strain of the virus of poliomyelitis..... 625
LANDSTEINER, K. Experiments on anaphylaxis to azoproteins.. 631
CORI, GERTY T. The effect of x-ray on the skin of vitally stained white mice. Establishment of an x-ray unit for the skin of the mouse..... 639
BROWN, WADE H., and PEARCE, LOUISE. Penetration of normal mucous membranes of the rabbit by *Treponema pallidum* and the influence of this mode of infection upon the course of the disease. Plates 33 and 34..... 645
OPIE, EUGENE L. The fate of antigen (protein) in an animal immunized against it..... 659

BINGER, CARL A. L., and BROW, GEORGE R. Studies on the respiratory mechanism in lobar pneumonia. A study of lung volume in relation to the clinical course of the disease. Plates 35 and 36 677

ANDERSON, HILDING C. The relation of blood pressure to the amount of renal tissue 707

DU NOÜY, P. LECOMTE. Surface tension of serum. VIII. Further evidence indicating the existence of a superficial polarized layer of molecules at certain dilutions. Plate 37 717

JONES, F. S. The influence of resistance on the character of disease in experimental respiratory infection 725

AVERY, OSWALD T., and NEILL, JAMES M. Studies on oxidation and reduction by pneumococcus. IV. Oxidation of hemotoxin in sterile extracts of pneumococcus 745

NEILL, JAMES M., and AVERY, OSWALD T. Studies on oxidation and reduction by pneumococcus. V. The destruction of oxyhemoglobin by sterile extracts of pneumococcus 757

No. 6, JUNE 1, 1924.

RIVERS, THOMAS M., and TILLETT, WILLIAM S. Further observations on the phenomena encountered in attempting to transmit varicella to rabbits. Plate 38 777

JONES, F. S., and LITTLE, RALPH B. The transmission and treatment of infectious ophthalmia of cattle 803

MELENEY, FRANK L., and ZAU, ZUNG-DAU. The viability of hemolytic streptococcus in certain solutions containing gelatin 811

COWDRY, E. V., and NICHOLSON, F. M. An histological study of the central nervous system in experimental *botulinus* poisoning 827

WEBSTER, LESLIE T. The epidemiology of a rabbit respiratory infection. I. Introduction 837

WEBSTER, LESLIE T. The epidemiology of a rabbit respiratory infection. II. Clinical, pathological, and bacteriological study of snuffles 843

WEBSTER, LESLIE T. The epidemiology of a rabbit respiratory infection. III. Nasal flora of laboratory rabbits..... 857
WEBSTER, LESLIE T. Microbic virulence and host susceptibility in paratyphoid-enteritidis infection of white mice. IV. The effect of selective breeding on host resistance..... 879
LINDER, G. C., LUNDSGAARD, C., and VAN SLYKE, D. D. The concentration of the plasma proteins in nephritis..... 887
LINDER, G. C., LUNDSGAARD, C., VAN SLYKE, D. D., and STILLMAN, E. Changes in the volume of plasma and absolute amount of plasma proteins in nephritis..... 921
HILLER, A., LINDER, G. C., LUNDSGAARD, C., and VAN SLYKE, D. D. Fat metabolism in nephritis..... 931
INDEX TO VOLUME XXXIX..... 957

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ELECTROCARDIOGRAPHIC EVIDENCE OF MYOCARDIAL INVOLVEMENT IN RHEUMATIC FEVER.

By ALFRED E. COHN, M.D., and HOMER F. SWIFT, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

Plates 1 to 3.

(Received for publication, July 23, 1923.)

It was Wells (1), a native of South Carolina, who recorded the circumstances in which rheumatism came to be associated with the development of heart disease. According to Wells, Dr. David Pitcairn was the first to point out this relation. He began to be interested in and to talk about it at St. Bartholomew's Hospital as early as 1788. Baillie (2)[1] appears to have been the first to refer to this teaching of Pitcairn's. Stimulated by Pitcairn's observations and Baillie's reference, Wells published[2] an account, illustrated by case reports, of rheumatic heart disease which leaves little doubt that he appreciated the connection between the two conditions. Later, after the introduction of the method of auscultation, it became possible during the life of patients to ascertain whether the rheumatic process involved the cardiac valves. It was probably[3] Dr. James Hope (3) who realized (1832) this possibility. Among the exciting causes of diseases of the valves, he specified "inflammation of the internal membrane of the heart, resulting from carditis, pericarditis—especially rheumatic, from fever or from any other cause."[4] That the heart muscle and the pericardium were in all likelihood also part of the process was in general assumed, but it remained for Aschoff and Geipel to demonstrate that rheumatic fever is accom-

[1] Baillie (2), p. 46.

[2] By this time accounts based on Baillie's report had been published also by Odier and by Dundas.

[3] There is an interesting controversy concerning this subject to be found in the 3rd edition of Hope's book, and in Bouillaud's Traité (1835).

[4] Hope (3), p. 319.

panied by a specific lesion and that this lesion is to be found in the heart muscle of patients dying of the disease. In the course of years this association has become so well recognized as to require no further emphasis.

Whether heart disease is likely to become established in an individual suffering from rheumatic fever, is still impossible to say during the course of the acute stage of the infection. There are no criteria for arriving at a judgment, even in the case of apparent affection of the cardiac valves. It often requires, indeed, the passage of long periods of time, sometimes of years, before the issue becomes apparent. The making of prognoses rested on no consideration beyond the state of the valves until 30 years ago, when the functions of the heart muscle came to be analyzed in detail. Since then the number of functions and mechanisms that can be distinguished and recognized has increased and has made possible the description of kinds of derangement not possible at an earlier period. In terms of these more newly described mechanisms observers now recognize injury to the heart not only at earlier stages in the course of several diseases, but have come also to describe types of injury hitherto quite unsuspected.

We have utilized the methods more recently introduced for investigating the effect on the heart of the process of rheumatic fever. The function of the heart to which we paid attention especially during this study is that of conduction between the auricles and the ventricles. But we have also utilized the fact that the general form of the electrocardiogram is, relatively speaking, constant in normal persons over prolonged periods of time. Under these circumstances, the occurrence of deviations from the contour of the usual curve becomes a sign of value. For the inference is clear that if the outline of the curve changes an alteration must have occurred in the heart to bring about this result. These signs, conduction time and curve form, have been employed before now as indications of change in the behavior of the heart, not only in rheumatic fever, but in other conditions as well.

As the result of a number of reports it is now well known that disturbance of conduction, and especially of heart block, occurs in the course of rheumatic fever (4). It may indeed be the first sign to

indicate the presence of the disease (5). Heart block has been observed at various stages in the febrile portion of its course and not uncommonly in the post febrile and convalescent periods. How often transient disturbances of conduction have led to permanent heart block and to heart failure in the later stages of the clinical history is not known. The interval may be brief, for occasionally (6), even in the early period of the rheumatic process, involvement of the heart leads promptly to death.

In the present enquiry we were not concerned with the problems of the late history of cases of cardiac involvement, but rather with the frequency with which the heart shares in the rheumatic process. We have made a systematic study in 37 patients.[5] The changes we have studied are chiefly, as has been said, of two sorts; first, alterations in the duration of the auriculoventricular conduction interval, and second, alterations in the form of the ventricular portion of the electrocardiogram.[6] Changes in the mechanism of the heart beat were observed in a number of patients. On these we will comment incidentally.

The cases are divisible into three groups; first, 17 cases preponderantly arthritic in type in the first attack of the disease; second, 12 cases also preponderantly arthritic but which had suffered at least one attack before coming under observation; and 8 cases suffering from the recurrent cardiac type of the affection. Although the last group of cases is classified as cardiac, arthritis was a feature in each case at some time during the period of observation. The changes we describe were not associated with the exhibition of drugs, such as digitalis, salicylates, or neocinchophen, except in those instances to which special reference is made.

The conduction time (auriculoventicular, P–R interval) was taken as normal for each individual when for a number of days in a febrile or

[5] Curves were taken of the same patient as often as every 24 hours; in some cases at intervals somewhat longer. 2,591 curves were taken in all.

[6] The events in which we were interested have been charted in a condensed form so that changes in the behavior of these functions might be correlated. Of the data recorded, only the temperature, the rate of the heart beat, and the conduction time are described in this report. The method of charting consists in joining, by vertical lines, the highest and lowest figures for the day. The upper limit of normal temperature is taken to be 99.6°F.

post febrile period, a constant level was maintained or when on subsequent examinations after discharge from hospital, this figure was obtained from electrocardiograms. It is of course not impossible that even with these precautions the normal conduction time for a given person was not obtained; the possibility of doing so may have disappeared with the onset of the disease.

Change in the duration of auriculoventricular conduction was a frequent, almost a constant occurrence; but we have noticed that the alterations, although frequent, did not run a course parallel with or corresponding to the temperature. We noticed in one patient (Text-fig. 1) in whom the conduction time was 0.24 second on the 16th day of disease when the temperature was 103.5°F., that 4 days later, when the temperature had fallen to 100°F., a marked increase in its duration took place to the extent that dropped beats (incomplete heart block) were observed. At that time she was taking neocinchophen. It is inferred that neocinchophen was not responsible for this occurrence because of the fact that later, although the exhibition of the drug was continued, the conduction time fell to limits which, for this patient, were considered normal. Parkinson (4) has made similar analyses of the relation of antirheumatic drugs to cardiac arrhythmias. When the temperature rose subsequently to 101° there was no further alteration in the conduction time. In another patient (Text-fig. 2) on the 6th day of disease, when the temperature was about 103.5°, the conduction time was within limits usual for him. Subsequently (24th day of disease) during a period of falling temperature the conduction time rose to 0.20 second. And later still (43rd day of disease) during an irregular febrile movement the conduction time attained its greatest elevation. Similar relations or lack of correlation were seen in the case of other patients (Text-fig. 3).

A close correspondence between these curves was seen, however, not infrequently. In one patient (Text-fig. 4) even when the elevation in temperature was slight, an increase in the duration of conduction was observed (end of March, beginning of May and June). In the May attack, when the conduction time was the longest recorded, the temperature reached only about 100.2°. At the same time there was, however, a burst of rapid heart action. Two other attacks of tachycardia were seen in this patient, each time associated with a

period of prolonged conduction time. In still another patient (Text-fig. 5) the correspondence in behavior of the two curves was striking, especially for about the first 100 days of the disease. Later, with or without fever, there was more or less continuous prolongation of the conduction rate.

These cases illustrate the changes to which we wish to call attention. In the 37 cases now reported we encountered it 31 times altogether, 24 times when fever was present, 15 times when it was absent. In 5 patients it was found in both associations. In 8 patients alteration in conduction did not end with increase in the P–R interval, but went on to the stage in which ventricular beats occasionally dropped out or to incomplete heart block (Tables I, II, and III).

The alteration which we have described does not include prolongation of conduction in the two main branches of the auriculoventricular system or its subdivisions. We do not distinguish between defects in the main divisions (bundle branch) and defects in the terminal arborizations (arborization block). Of such alterations we saw three examples. These were all transient.

The Q R S Period.—We describe next alterations in the ventricular portion of the electrocardiogram. Of these we distinguish three main varieties; first, that involving the Q R S complex; second, that involving the R–T period; and third, that affecting the T wave itself. Of changes which occur with respiration, for example, in the height of the R (or S) wave in Lead III, we have numerous examples. To these we wish merely to refer. They occur naturally when the direction of potential is nearly perpendicular to a lead or when, in the rotation of the heart in the course of contraction, a new electrical potential is projected on the frontal plane. A change of a different nature is seen in another series of curves (Fig. 1). The curve taken on October 3, 1922, may be taken as a control. In the curve taken on June 2, the S wave in Lead III, usually absent, is much increased, while the R wave, usually prominent, is decreased. There is, moreover, a notch in the R waves. Transient changes like this are probably uncommon and are probably significant. We attach no especial importance to changes which affect the amplitude of the waves, for rather striking changes are not infrequent even in quite normal

persons. Apparently when the R waves in Lead III are split, there is the greater tendency to take on these varient forms.

A change in the R waves of much interest is one which we observed in two cases. In one case (Text-fig. 6 and Fig. 2) it consists of an

TABLE I.

Cases in the First Attack of Rheumatic Fever Showing Especially Polyarthritis.

Case No.	Sex and age.	Increase in P-R time.*		Change in Ekg.	Irregularities.	Valvular disease.	Pericarditis.†	Subcutaneous nodules.†
		Febrile.	Non-febrile.					
	yrs.							
1‡	F. 17	+	0	+	I.H.B.; A.P.C. blocked.§	?‖	+	0
2	M. 21	+	+	+	V.P.L.	?	0	0
3	F. 30	+	0	+		M.I.**	0	0
4	F. 30	0	0	+	S.I.; V.P.L.	M.I.	0	0
5	M. 31	0	0	0	V.P.L.; S.I.	0	0	0
6	M. 14	0	+	+		M.I.	0	0
7	M. 19	+	+	+		?	0	0
8‡	M. 26	+	0	+	V.P.L.	?	0	0
9	F. 30	0	+	+	S.I.; V.P.L.	?	0	0
10	M. 33	+	0	+	A.F.; V.P.L.; V.P.C.L.	M.I.	0	0
11	M. 17	0	+	+	A.P.C.	?	0	0
12‡	F. 31	+	0	+	A.F.; A.Fl.; I.H.B.; V.P.L.; V.P.C.R.; I-V Block.	?	+	0
13	M. 23	0	+	+	I.H.B.; A.P.C.	?	0	0
14	M. 14	+	+	+	A.P.C.; V.P.C.R.	M.I.	+	0
15	F. 25	0	+	+		?	0	0
16	M. 19	+	+	+	I.H.B.; P.C.	?	0	0
17	F. 29	+	+	+		M.I.	0	0

* An increase of 0.02 second or more.

† In this and the following two tables + represents the presence of this lesion.

‡ Indicates special charts.

§ The abbreviations in this column are arranged alphabetically. A.F. indicates auricular fibrillation; A.Fl., auricular flutter; A.P.C., auricular premature contractions; I.H.B., incomplete heart block; I-V Block, intraventricular block; P.C., premature contractions; S.I., sinus irregularity; V.P.C.L., ventricular premature contractions left; V.P.C.R., ventricular premature contractions right; V.P.L., ventricular preponderance left.

‖ Indicates in this and the two following tables that the presence of valvular disease is doubtful.

** In this and the two following tables M.I. represents mitral insufficiency.

TABLE II.

Cases of Recurrent Attacks of Rheumatic Fever Exhibiting Especially Polyarthritis.

Case No.	Sex and age.	Increase in P-R time.* Febrile.	Increase in P-R time.* Non-febrile.	Change in Ekg.	Irregularities.	Valvular disease.	Pericarditis.	Subcutaneous nodules.
	yrs.							
18	M. 14	0	0	+		+† M.I.; M.St.	+	0
19	F. 38	+ −	0	+	A.F.;A.P.C.;+P-R;I.H.B.; V.P.R.; V.P.C.R.‡	+ M.I.; M.St.; A. rough.	+	0
20	M. 12	+ 0	0	+	S. I.	+ M.I.	0	0
21	F. 19	+ −	0	+	V.P.C.R.; V.P.L.	+	+	+
22	M. 14	+ −	+	+	A.P.C.; Atyp. A.P.C.	+ M.I.; A.I.; A. rough.	0	0
23§	M. 8	+	0	+	V.P.C.; I.H.B.; A.P.C.; A-V.P.C.; V.P.C.L.	+ M.I.; M.St.	0	+
24	M. 22	0	0	+	V.P.L.; A.P.C.	+ M.I.; A.I.?	0	0
25	F. 22	0	+	+	V.P.L.? A. Par. Tachy.; S.I.; Atyp. Ekg.; transition to normal.	?	0	0
26§	M. 52	+ 0	0	+	A-V Rhythm; A. Par. Tachy.; V.P.L.	? M.I.	0	0
27	M. 15	+ 0	0	+	V.P.C.R.; V.P.R.	+ M.I.	0	0
28	M. 15	+ 0	+	+	A-V Rhythm; runs of nodal P.C.; A-V.P.C.	+ M.I.	0	0
29	M. 17	+	0	0		+ M.I.; A.I.?	0	0

* An increase of 0.02 second or more.

† In this and the following table M.St. represents mitral stenosis; A. rough., aortic roughening; A.I., aortic insufficiency.

‡ For abbreviations in this column see Table I. Additional ones are given as follows: + P-R, prolonged auriculoventricular interval; A-V.P.C., auriculoventricular premature contractions; A.Par.Tachy., auricular paroxysms of tachycardia; Atyp. Ekg., atypical electrocardiogram; A-V Rhythm, auriculoventricular rhythm; V.P.R., ventricular preponderance right.

§ Indicates special charts.

TABLE III.

Cases of Rheumatic Fever Predominantly Cardiac but Exhibiting Also Arthritic Involvement.

Case No.	Sex and age.	Increase in P-R time.*		Change in Ekg.	Irregularities.	Valvular disease.	Pericarditis.	Subcutaneous nodules.
		Febrile.	Non-febrile.					
	yrs.							
30	M. 13	+		+	V.P.L.; V.P.C.R.; +P-R.; I.H.B.; Atyp. Ekg.†	+ M.I.; M.St.	+	+
31	M. 8	+ 0	+	+	V.P.L.; Tachy.	+ M.I.; A.I.; A. rough.	0	0
32	M. 14	+		+	V.P.L.; I-V Block; A.F.? Tachy.	+ M.I.; A.I.; A. rough.	0	0
33	M. 11	+	0	+	V.P.R.	+ M.I.; A.I.?	+	0
34	M. 7	0	0	+	A.P.C.	+ M.I.; A.I.?	0	0
35	F. 9	0	+	+	V.P.R.; S-A Block; I.H.B.; V.P.C.R.; V.P.C.L.; A.F.	+ M.I.; M.St.	+	+
36‡	F. 17	+		+	+P-R; A-V Rhythm; Atyp. Ekg.	+ M.I.; A.I.; Tr. I.;§ Tr. St.	+ M.I.; A.I; Tr. I.; Tr. St.	0
37	F. 41	0‖	0	+	A.P.C.; A-V.P.C.; S.I.; A-V Rhythm; I-V Block; Interpolated V.P.C.R.; A-V S-A Rhythm.	+ M.I.; A.I.?	0	0

* An increase of 0.02 second or more.

† For abbreviations in this column see Tables I and II. Additional ones are given as follows: S-A Block, sinoauricular block; A-V S-A rhythm, auriculoventricular and sinoauricular rhythm.

‡ Indicates special charts.

§ Tr. I. indicates tricuspid insufficiency; Tr. St., tricuspid stenosis.

‖ This measurement did not increase even in an attack of influenza.

interruption of the down stroke of the R wave in Lead I (January 31) and the beginning, immediately at the point of interruption, of a wave convex upward occupying the whole R–T period. In its usual form, the foot points of the R wave are to be found on almost the same abscissa. This deformity persisted for several days; later (March 2) the shape of the wave returned to the initial state. A change of a similar nature was found in the second patient in Lead III, on December 1 and 10 (Fig. 3). In this case, the prominent phase of the Q R S complex was the S wave. The up stroke of this wave overshot the level of the foot point of the R wave, so that the R–T period lies at a level considerably higher than that of the P–R interval or of the diastolic (T–P) period.

The significance of this phenomenon is by no means clear. Similar changes have been noticed in conditions other than rheumatism. It has been described by Pardee (7) as evidence of blocking of a branch of the coronary artery. In other cases of coronary thrombosis Wearn (8) failed to find this sign or a sign which Pardee described as its sequel. It is, however, not remarkable that appearances like this are found. Their discovery should be expected in several circumstances for the form of the curve is probably not characteristic of the nature of the injury, but is the result rather of any injury involving a given area. The contour of the curve probably is an indication of disturbance of certain functions of the muscle, and is perhaps dependent on the site in the heart wall at which the injury has taken place.

It is important to notice that these disturbances are transient and indicate the fleeting nature either of the injury or of its effect. They are entirely comprehensible in cases of rheumatic fever in which the ephemeral character of certain of its manifestations, whether in the joints, the skin, or in the other tissues which are frequently involved, is well known. The inference it seems to us is, therefore, justified that the nodule described by Aschoff, which is its basic lesion in the heart, and perhaps the ischemic areas consequent upon blood vessel involvement, underlie the electrical disturbance to which the injured muscle gives rise. As to how extensive these lesions need be in order to bring about this result or in what situation in the heart they must be located are points on which we can make no

have an effect on rate similar to work, for in lobar pneumonia (12) elevation in rate is almost constantly found, while increase in auriculoventricular conduction is relatively speaking infrequent.

In the measurements made of the length of the auriculoventricular interval, variations were found, as has been said, frequently. The variations occurred in the same individual, not only in the same curve, but also from day to day. The precaution was taken to make the measurements in Lead II. These variations were, we presumed, due to the rheumatic process. What variations to expect in normal persons we did not know. We have accordingly studied the behavior of this portion of the curve in 6 normal persons. Electrocardiograms were made synchronously of the 3 leads, using 3 galvanometers. The respiratory movements were inscribed on the curves

TABLE IV.

*The Duration of the Auriculoventricular Interval in Three Normal Persons in Inspiration and Expiration.**

Case No.	Inspiration.		Expiration.		Difference between average inspiration and expiration.
	Average.	Greatest range.	Average.	Greatest range.	
1	0.1369	0.0203	0.1302	0.0361	0.0067
2	0.1932	0.0214	0.1862	0.0152	0.0070
3	0.1507	0.0674	0.1395	0.0476	0.0112

* The measurements were made by means of a Lucas comparator.

according to a method described elsewhere (13). In the case of these normal persons we obtained data showing the variations which occurred from day to day and also the difference which took place between inspiration and expiration (Table IV).

The range in the first two cases is 0.02 second in inspiration; and 0.03 and 0.015 second in expiration. In Case 3, the range is much greater, 0.0674 second in inspiration and 0.0476 in expiration. This range (Case 3) is wide and far greater than one would expect if one used our entire experience as the basis of judgment. The reason for it must be quite exceptional. On the whole, the average of the inspirations is a trifle greater than that of the expiration. On any given day one measurement (inspiration) may be greater than that of the other or the same. That there is a daily variation is in itself

a matter of interest. These figures furnish information on the degree of fluctuation which the factors that are concerned in the production of these events experience. Among these are such factors as vagus tone, and the presence of an inflammatory lesion. It is not surprising, therefore, in the light of these data, to find differences from day to day in the curves of rheumatic patients. The daily records did not of course include curves of respiration; but even if they had, considerable difference in the same phase of breathing, as we have shown, would have been found. In the light of these facts we are, it seems to us, justified in placing significant changes in conduction at a figure at or above 0.02 second.

It is not the purpose in this paper to discuss the question whether it is possible from studying these curves to conclude which portion of the heart is involved in the rheumatic process; nor whether the nature of the process is toxic or structural; nor whether the alterations are specific for rheumatic fever; nor whether the injury to the heart indicated by the alterations in the curves is transient or permanent. To us it appears that, for the moment, none of these questions is solvable. The alterations found in this series of curves have, however, led us to investigate the constancy of the electrocardiogram in a single person. For this reason we have examined series of 8 to 10 curves taken over periods of about 14 days of the 6 persons already mentioned, with the view to ascertaining how uniform over limited periods of time the electrocardiogram of a single person actually is (13). In each series taken from day to day alterations were in fact found. But they were slight, quantitative in nature, not qualitative such as those one recognizes as quite different and distinct forms.

If the conclusion is correct that the form of the electrocardiogram does not change either in conduction or in contour in normal individuals in the sense in which it does in rheumatic fever, it is probable that a process of some nature is operative in the heart of individuals infected with rheumatic fever. Whether the alteration in the heart to which the rheumatic process gives rise is specific either in the nature of the lesion or process, or in its location is at present a matter of secondary importance to us. It is our purpose now to indicate the frequency of its presence. In other infectious diseases such as lobar

pneumonia it is already known that changes in the curves do not take place; certainly not with the frequency with which they have been found in rheumatic fever. As a result of this study it is possible to emphasize the fact that in rheumatic fever we possess a means of knowing whether, and when, the heart is involved in the process. That the heart may become involved is a fact already well known. Further study will show whether information, obtained by this means represents changes in the heart of significance for prognosis.

TABLE V.

*Summary of Cardiac Irregularities.**

Premature contractions	1
Auricular premature contractions	10
Ventricular premature contractions, right	7
" " " left	3
Auriculoventricular premature contractions	2
Interpolated ventricular contractions	1
Sinoauricular block	1
Auriculoventricular block	8
Intraventricular block	3
Auricular fibrillation	5
" flutter	1
Auriculoventricular rhythm	4
Auricular paroxysms of tachycardia	2

* If the entire material had been searched, a greater number of instances of irregular heart action would undoubtedly have been found. But even this figure would not give an accurate account of the incidence; more than were photographed must have occurred.

It is unnecessary to report in detail the irregularities that were observed in the course of this study. A summary of them is given in Table V. We wish to point out that the number of cases of heart block, that is to say cases of extreme affection of the auriculoventricular system, was relatively speaking infrequent compared with the number of times this structure was found to be affected. Cases of transient auricular fibrillation were also encountered; in one of these we obtained a record of the transition to the normal mechanism.[7]

[7] Cohn, A. E., Irregularities, Nelson, Loose-leaf medicine, New York, 1920, iv, 329, Fig. 102.

Aside from the variety of irregularities, the number of cases in which they were observed, and the frequency with which they occurred in the same case, are striking. We point to these occurrences only to emphasize the fact that they represent contributary evidence of the fact of how often the heart is affected by the rheumatic process.

SUMMARY.

These observations then show that in one way or the other the heart was affected in 35 of 37 cases of rheumatic fever. The evidence was of three sorts; first, the duration of auriculoventricular conduction was increased, though not always, and usually not to the degree of causing heart block; second, alteration in the ventricular complex of the electrocardiogram affecting in certain instances the Q R S complex and in others either the interval between R wave and T wave, or the T wave itself; and third, the occurrence of numerous irregularities in cardiac rhythm.

These signs are characteristic of cardiac involvement, but are not specific for rheumatic fever; that is to say, from their presence rheumatic fever cannot be diagnosticated; all of them express merely derangement of the heart. They are signs which are, however, to be taken as evidence that the heart is affected by the rheumatic process even though an inference cannot be drawn as to the nature or the permanence of the injury. This may be slight and altogether transient even if the sign appears to be of advanced degree. The facts brought forward show that it is possible during the course of the disease to know whether the heart is involved in the general process. When sufficient data have been accumulated over a sufficiently long period of time, the usefulness for prognosis of observations like these will become established.

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Explanation of Text-Figs. 1–6.

Each day is represented by one vertical column.

The maximum and minimum temperature for a day is represented by the upper and lower end, respectively, of the line in the temperature chart; hence, the range of temperature for a day is indicated by a single line. All temperatures are taken by rectum, hence 99.6°F. is considered normal.

Range of pulse for a day represented in a similar manner.

Conduction time range represented by a vertical line and the upper point joined by a light line to show the variation from day to day.

Degree of arthritis: Each block represents one joint; a solid block indicates marked signs of inflammation; a dot, slight signs.

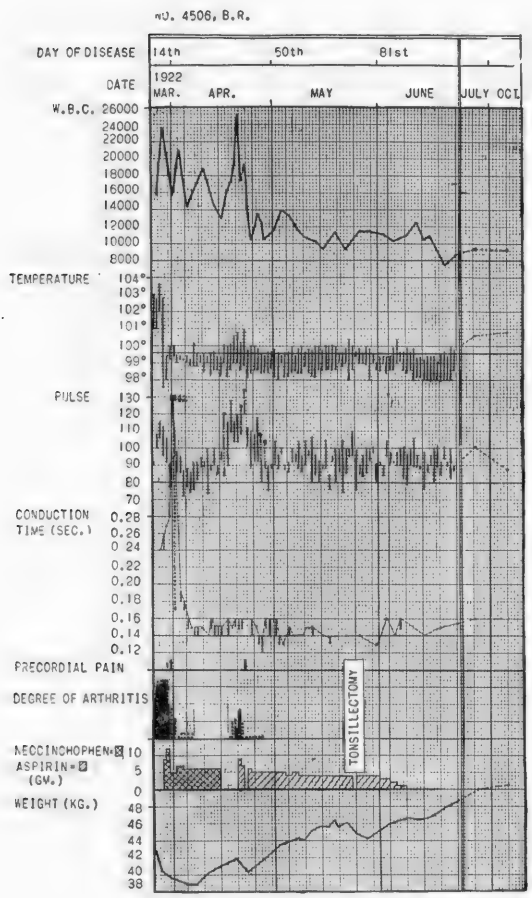
Precordial pain and tenderness: The length of a line indicates the degree of intensity of the respective symptom for 1 day.

Drugs: Sodium salicylate, aspirin, cinchophen or neocinchophen—each small square represents 1 gm. Digitan—each small square represents 0.1 gm.

W. B. C.: Range of leucocytosis.

Weight curve is self-explanatory.

Solid lines indicate observations made while patient was in the wards. Broken line indicates observations made at the time of return follow-up visits.


No. 4506, B.R.
DAY OF DISEASE
14th
50th
81st
DATE
1922
MAR.
APR.
MAY
JUNE
JULY
OCT.
W.B.C.
26000
24000
22000
20000
18000
16000
14000
12000
10000
8000
TEMPERATURE
104°
103°
102°
101°
100°
99°
98°
PULSE
130
120
110
100
90
80
70
CONDUCTION TIME (SEC.)
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
PRECORDIAL PAIN
DEGREE OF ARTHRITIS
TONSILLECTOMY
NEOCINCHOPHEN
ASPIRIN
(GM.)
10
5
0
WEIGHT (KG.)
48
46
44
42
40
38

TEXT-FIG. 1.

No. 4506.—B. R., female, age 17 years.

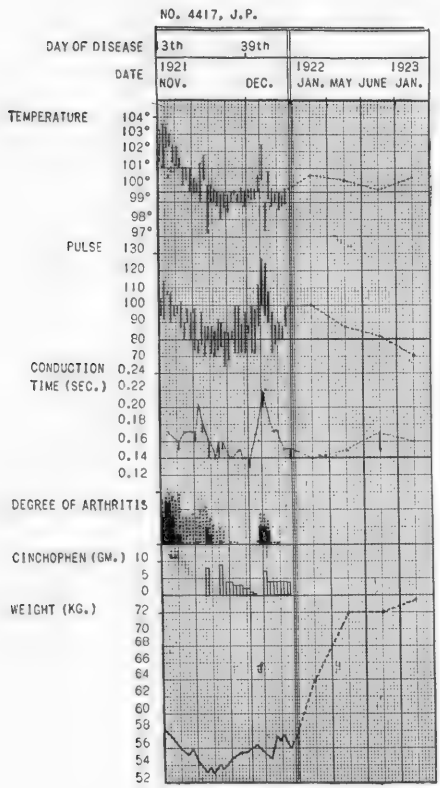
Admitted Mar. 26, 1922. Discharged June 24, 1922.

First attack of rheumatic fever.

Present Illness.—Began 13 days previously with anorexia, malaise, loss of weight, and pain in ankles; 4 days ago arthritis began to migrate to knees; 2 days ago, to arms.

Physical Examination.—Marked, extensive polyarthritis. Heart markedly irregular with groups of coupled and tripled beats. Apical murmur not transmitted.

Course.—(Text-fig. 1.) Day following admission regular cardiac rhythm with marked gallop character; prolongation of P–R time. Spread of polyarthritis and further increase of P–R time. Rapid disappearance of arthritis and fever following the administration of neocinchophen; but P–R time increased; partial heart block developed on the 4th day of administration; a transitory rub over the precordium. Drug continued; conduction time normal on the 25th day (Apr. 6); gallop rhythm disappeared. Continuing leucocytosis indicated a persistence of the infection in spite of lack of symptoms. Neocinchophen discontinued; a definite relapse of polyarthritis, fever, increase of heart rate, and gallop rhythm 2 days afterward. Symptoms and signs disappeared following large doses of aspirin; but the drug was continued for 50 days because of the persistent leucocytosis. Patient recovered with only persistent apical systolic murmur not transmitted; no new murmurs nor signs of cardiac insufficiency.


NO. 4417, J.P.
DAY OF DISEASE
13th
39th
DATE
1921
NOV.
DEC.
1922
JAN. MAY JUNE
1923
JAN.
TEMPERATURE
104°
103°
102°
101°
100°
99°
98°
97°
PULSE
130
120
110
100
90
80
70
CONDUCTION TIME (SEC.)
0.24
0.22
0.20
0.18
0.16
0.14
0.12
DEGREE OF ARTHRITIS
CINCHOPHEN (GM.)
10
5
0
WEIGHT (KG.)
72
70
68
66
64
62
60
58
56
54
52

TEXT-FIG. 2.

No. 4417.—J. P., male, age 26 years.

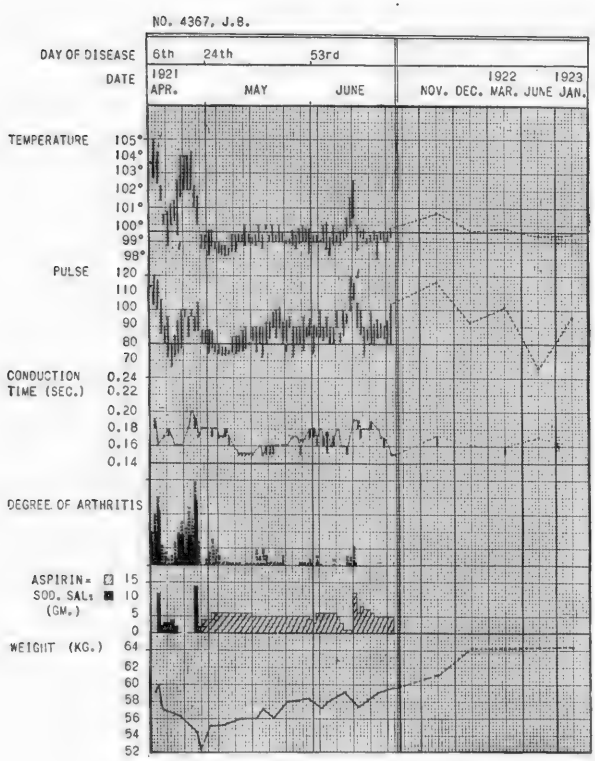
Admitted Nov. 5, 1921. Discharged Dec. 13, 1921.

First attack of rheumatic fever.

Present Illness.—3½ weeks before admission, sore throat, fever, malaise, followed by a cough, for 3 days. 12 days ago, polyarthritis and fever began and lasted 5 days. Following exertion, polyarthritis and fever recurred; 2 days before admission he was somewhat better.

Physical Examination.—Mild pharyngitis, extensive polyarthritis. Heart not enlarged. Soft precordial and apical systolic murmurs not transmitted.

Course.—(Text-fig. 2.) Without drugs, fever, pulse rate, toxicity, and polyarthritis diminished for 9 days; then a relapse occurred. Polyarthritis and fever preceded by an increase in P–R time (Nov. 24). Large doses of cinchophen followed by marked relief. Dose reduced and then discontinued; definite relapse followed consisting of fever, pharyngitis, polyarthritis, and a much higher pulse rate. P–R time again prolonged; precordial burning sensation during one night. Cardiac murmur no longer heard. Fever and symptoms again responded to cinchophen. Murmurs did not reappear. Patient left hospital against advice. Small doses of aspirin at home; no more relapses. No symptoms of cardiac insufficiency nor signs of valvular disease since recovery.


NO. 4367, J.B.
DAY OF DISEASE
6th
24th
53rd
DATE
1921
APR.
MAY
JUNE
1922
NOV. DEC. MAR. JUNE
1923
JAN.
TEMPERATURE
105°
104°
103°
102°
101°
100°
99°
98°
PULSE
120
110
100
90
80
70
CONDUCTION TIME (SEC.)
0.24
0.22
0.20
0.18
0.16
0.14
DEGREE OF ARTHRITIS
ASPIRIN=
SOD. SAL=
(GM.)
15
10
5
0
WEIGHT (KG.)
64
62
60
58
56
54
52

TEXT-FIG. 3.

No. 4367.—J. B., male, age 18 years.

Admitted Apr. 6, 1921. Transferred to Presbyterian Hospital, June 25. Discharged Sept. 11, 1921. Readmitted Apr. 18, 1923.

Previous Rheumatism.—First attack of rheumatic fever with migratory polyarthritis 4 years ago. Second attack 3 years ago; heart involved then. No cardiac insufficiency.

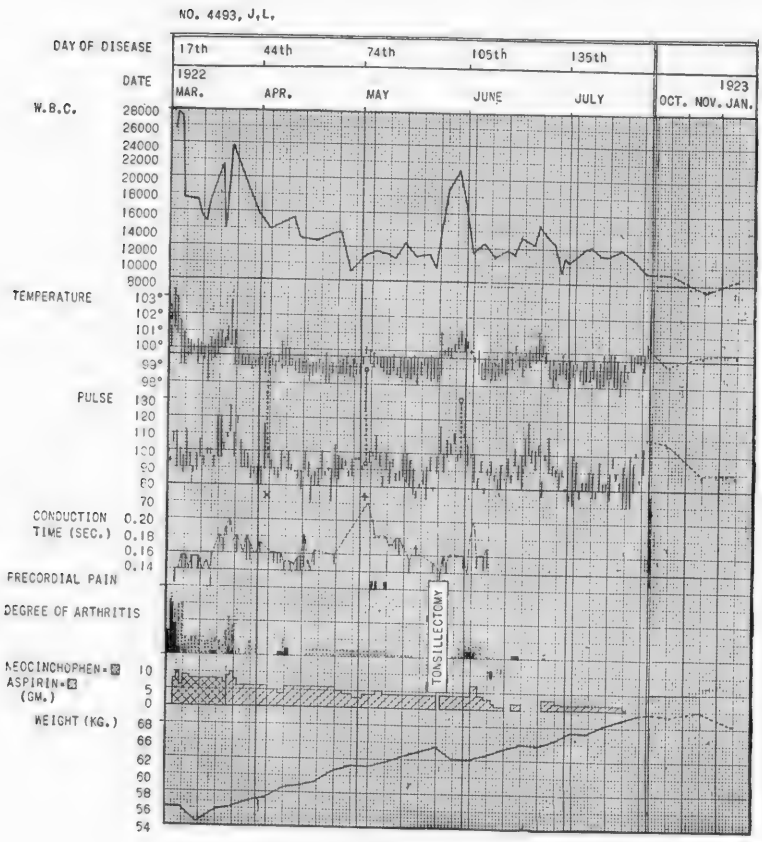
Present Illness.—4 weeks ago sore throat and fever of 104°F. In bed 1 week 2 weeks ago. Slight pain in left ankle. 5 days ago polyarthritis began; now increased in intensity and extent.

Physical Examination.—Pharyngitis, enlarged tonsils, polyarthritis. Heart: mitral stenosis and insufficiency; second pulmonic sound markedly accentuated.

Course.—(Text-fig. 3.) Pyrexia, toxicity, and polyarthritis decreased only during administration of sodium salicylate. Later, 5 gm. of aspirin were given daily. Occasional slight fever, increased pulse rate; mild arthritis. Relapse when aspirin was reduced to 1 gm. daily; disappeared following large doses of the drug. On admission and in relapses, P–R time increased. During June subcutaneous fibroid nodules in tendon sheaths of hands and over left elbow.

Patient transferred to Presbyterian Hospital. During his stay (11 weeks) several bouts of fever, polyarthritis, precordial pain, cardiac arrhythmia, and new subcutaneous nodules. Tonsillectomy.

From Nov., 1921, until Apr., 1923, well, except for signs of mitral disease. Apr. 16, 1923, fourth attack of rheumatic fever; symptoms, signs, prolonged P–R time, and course similar to that above recorded.



× Nodal rhythm. + Paroxysmal tachycardia.

TEXT-FIG. 4.

No. 4493.—J. L., male, age 52 years.

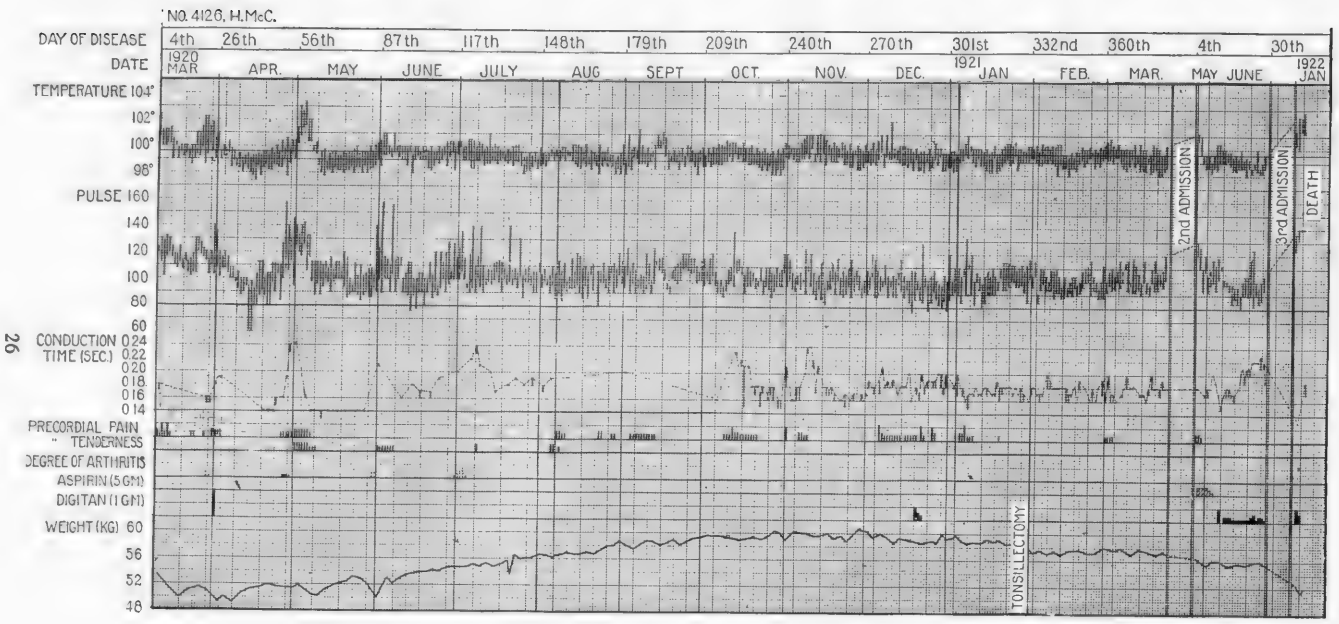
Admitted Mar. 5, 1922. Discharged July 25, 1922.

Previous Rheumatism.—Mild arthritis in ankles 2 years ago; duration 3 days. Sore throat for many years.

Present Illness.—Arthritis in ankles began 16 days ago, migrated to knees and hips; yesterday shoulders, elbows, and wrists were involved.

Physical Examination.—Conjunctivitis, rhinitis, pyorrhea alveolaris, buried tonsils, marked polyarthritis, emphysema. Cardiac apical systolic murmur.

Course.—(Text-fig. 4.) Polyarthritis and fever increased. Marked improvement after large doses of neocinchophen. Leucocytosis, slight fever, increased pulse rate, and stiffness in a few joints persisted. Relapse, accompanied by increased P–R time occurred while taking 8 gm. of neocinchophen a day. Aspirin substituted for neocinchophen with marked benefit. Attack of nodal tachycardia Apr. 3. Leucocytosis, increased pulse rate, and slight stiffness of shoulders persisted. Aspirin decreased; May 2, auricular tachycardia. Afterward increased P–R time, precordial pain, and slight fever. Later, while under influence of aspirin signs of infection except leucocytosis and increased heart rate disappeared. Apical systolic murmur and occasional gallop rhythm persisted. May 24, tonsillectomy. Ekg. during operation normal. After tonsillectomy, arthritis and tachycardia (short attack), followed in 3 and 4 days by increased conduction time. In June, mild relapse with rapid heart action; apical systolic murmur rougher. Joints baked, active and passive motion, and progressive exercise, followed by improvement. Since discharge, no symptoms of cardiac insufficiency; occasional stiffness in joints, persistence of apical systolic murmur, not transmitted, but auscultatory diagnosis obscured by pulmonary emphysema.


No. 4126. H.McC.
DAY OF DISEASE
4th 26th 56th 87th 117th 148th 179th 209th 240th 270th 301st 332nd 360th 4th 30th
DATE
1920 MAR APR. MAY JUNE JULY AUG SEPT OCT. NOV. DEC. 1921 JAN FEB. MAR. MAY JUNE 1922 JAN
TEMPERATURE 104° 102° 100° 98°
PULSE 160 140 120 100 80 60
CONDUCTION TIME (SEC.) 0·24 0·22 0·20 0·18 0·16 0·14
PRECORDIAL PAIN
" TENDERNESS
DEGREE OF ARTHRITIS
ASPIRIN (5 GM.)
DIGITAN (1 GM.)
WEIGHT (KG) 60 56 52 48
TONSILLECTOMY
2nd ADMISSION
3rd ADMISSION
DEATH

TEXT-FIG. 5.

No. 4126.—H. McC., female, age 17 years.

Admitted Mar. 10, 1920. Discharged Mar. 24, 1921. Readmitted May 28, 1921, and discharged June 23, 1921. Readmitted Jan. 20, 1922. Died Jan. 24, 1922.

Previous Rheumatism.—First attack 3 years ago; duration 3 months. Tonsillitis every winter for many years frequently accompanied by quinsy. Tonsillitis 2 months ago. 7 weeks ago told of presence of heart trouble. Since then slight precordial pain on exertion. Light work until 2 weeks ago.

Present Illness.—Began 3 days ago with mild polyarthritis for 1 day. 2 days ago severe precordial pain; now increased with palpitation and dyspnea.

Physical Examination.—Patient showed orthopnea, rapid respirations, distinct pallor, enlarged tonsils, mild pharyngitis. Heart enlarged to right and left. Gallop rhythm, blowing apical systolic murmur, accentuated second pulmonic sound, and pericardial friction rub persisting 7 days.

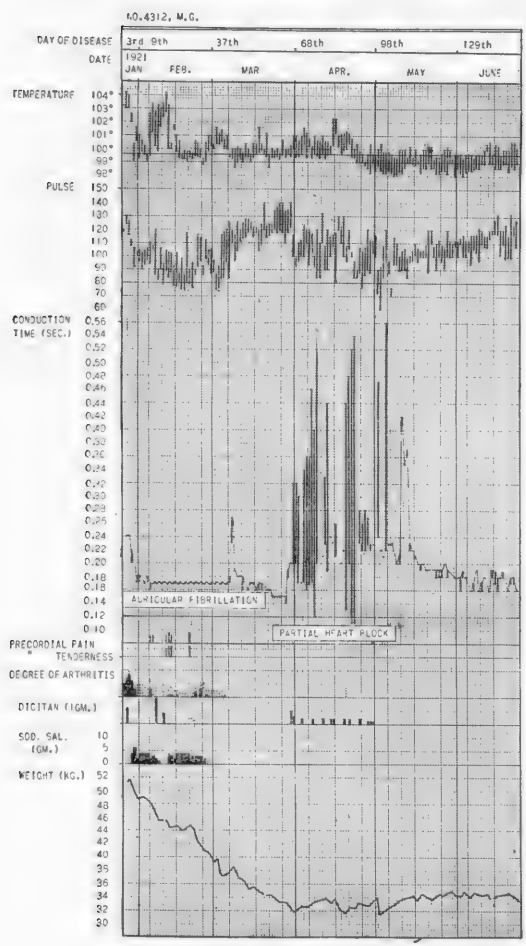
Course.—(Text-fig. 5.) For 2 months, relapses with fever, high pulse rate, precordial pain, increased P–R time, and mild arthritis. Next 2 months relapses with less fever, but paroxysms of tachycardia, prolonged P–R time, and mild arthritis. Subsequent relapses during 1st year were cardiac, characterized by paroxysms of tachycardia, precordial pain and tenderness, gallop rhythm, and prolonged P–R time. During the first 8 months relapses about every 4 weeks; later more irregular. Aortic diastolic and presystolic apical murmurs sometimes heard. Later (Jan., 1921) constant aortic systolic murmur. Jan. 31, 1921, tonsillectomy. Exercise was started Feb. 15, 1921, and gradually increased. Mar. 1, slight precordial pain lasting 4 days. Aortic diastolic murmur not heard for some time now heard again. Mar. 24, discharged, feeling well although mild fever and some increase in pulse rate were present; also gallop rhythm, apical systolic murmur, aortic systolic murmur, a diastolic murmur over upper sternum, and soft systolic blowing murmur over the lower sternum to the ensiform.

At home Mar. 24 to May 28. Well the 1st week, then mild polyarthritis, "cold" in the chest, and attack of palpitation. Another cold the middle of May; rapid heart and precordial pain; herpes labialis. May 15 to 28, in bed with constant precordial pain and dyspnea.

Second Admission.—May 28 to June 23, 1921. Temperature 101°, pulse rate 120 to 130, precordial pain, gallop rhythm, and apical systolic murmur. June 6, aortic diastolic murmur again heard; digitan June 6; some slowing of pulse. Discharged June 23.

At home June 23, 1921 to Jan. 20, 1922. Aug. 7 to 21, relapse with precordial pain radiating down left arm and into left side of neck; fear of impending death. Until Sept. 20, better and no pain. Sept. 20, gallop rhythm, aortic diastolic and apical systolic murmurs present.

Readmitted Jan. 20, 1922. Marked cardiac decompensation. Edema of extremities, fluid in abdomen and chest, and in knee joints. No precordial pain, tenderness, or hyperalgesia. Apical systolic murmur, gallop rhythm, systolic and diastolic blowing murmurs over the aortic area. Rhythm regular. Liver enlarged. Digitan given without effect. The patient died suddenly Jan. 24, 1922.


M.O. 4312, M.G.
DAY OF DISEASE
3rd 9th 37th 68th 98th 129th
DATE
1921
JAN FEB. MAR APR. MAY JUNE
TEMPERATURE
104° 103° 102° 101° 100° 99° 98°
PULSE
150 140 130 120 110 100 90 80 70 60
CONDUCTION TIME (SEC.)
0.56 0.54 0.52 0.50 0.48 0.46 0.44 0.42 0.40 0.38 0.36 0.34 0.32 0.30 0.28 0.26 0.24 0.22 0.20 0.18 0.16 0.14 0.12 0.10
AURICULAR FIBRILLATION
PARTIAL HEART BLOCK
PRECORDIAL PAIN
TENDERNESS
DEGREE OF ARTHRITIS
DIGITAN (IGM.)
SOD. SAL. (GM.)
10 5 0
WEIGHT (KG.)
52 50 48 46 44 42 40 38 36 34 32 30

TEXT-FIG. 6.

Postmortem Diagnosis.—Adherent pericardium; hypertrophy and dilatation; chronic thickening of the tricuspid, mitral, and aortic valves; verrucous endocarditis of the tricuspid, mitral, and aortic valves. Chronic fibrinous pleurisy; chronic passive congestion of the liver, spleen, and kidneys. Ascites. Edema of legs.

Microscopic Examination.—Numerous Aschoff bodies throughout myocardium, mural endocardium, and pericardium; inflammation of tricuspid, mitral, and aortic valves and of chordæ tendineæ of mitral and tricuspid valves. Endarteritis and thrombosis of fine branches of coronary artery.

No. 4312.—M. G., female, age 31 years.

Admitted Jan. 26, 1921. Discharged June 24, 1921.

First attack of rheumatic fever.

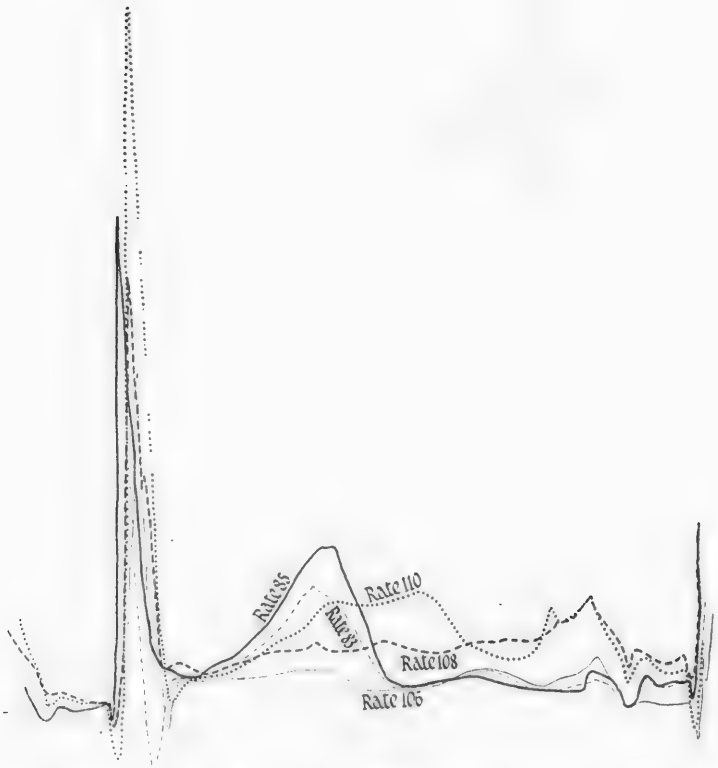
History.—Tonsillitis 1 month ago; in bed 10 days. Following this, loss in weight (20 pounds), occasional pain in ankles, and dyspnea on climbing stairs. 2 days ago chills and fever, 1 day ago, pain in ankles; arthritis spread to knees and lumbar spine the day of admission; *i.e.*, 3rd day of disease.

Physical Examination.—Redness of pharynx, polyarthritis involving joints of leg and lumbar spine. Heart enlarged. Numerous premature contractions. Rough systolic and short diastolic rubbing sound over base of heart. Prolonged P–R time.

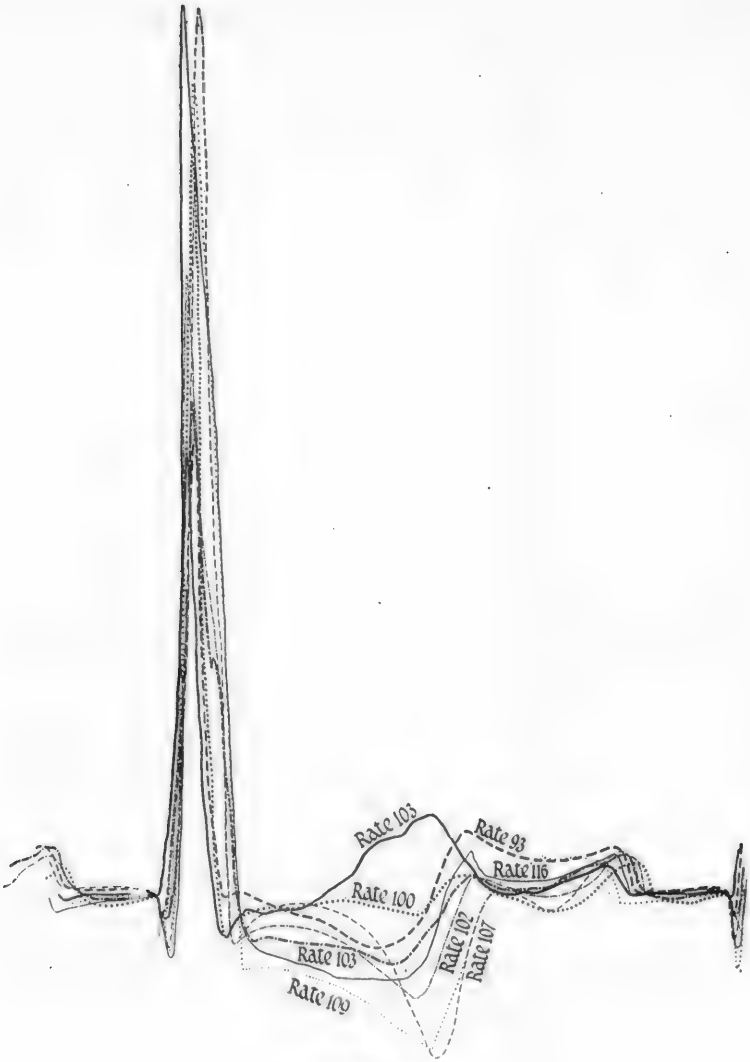
Course.—(Text-fig. 6.) Marked intoxication; spread of arthritis on 4th and 5th days. Extrasystoles disappeared, murmurs changed to rough systolic over base and soft systolic blow at apex; P–R time somewhat shorter on 6th day. Sodium salicylate with marked benefit; arthritis diminished; fever less.

12th day yellowish pus expressed from both tonsils. 13th to 26th day, "to and fro" murmurs heard the 4th and 5th days again present; now have character of pericardial friction. Systolic blowing murmur over aortic area. Dose of sodium salicylate reduced because of nausea and vomiting. 14th day, recurrence of high fever, increase of arthritis, total cardiac irregularity (auricular fibrillation), and marked pulse deficit. 40th and 41st days, auricular flutter. Doses of sodium salicylate were small because of nausea; drug discontinued because of vomiting. 26th day, signs of pericardial effusion; and double pleurisy with effusion from 17th to 35th days. Arthritis disappeared 2 days before cessation of auricular fibrillation. For next 3 weeks rapid heart action with low grade fever; no arthritis; continuous loss of weight due in part to anorexia and nausea. Small doses of digitan given for 5 weeks because of tachycardia caused partial heart block with reduction in heart rate and improvement in general condition. May and June, occasional slight febrile relapse. Anorexia and vomiting troublesome. Short rough systolic murmur almost continuously over base of heart, loudest over sternum, and soft blowing apical systolic murmur from time to time. On discharge there was merely a clicking systolic sound at base to left of sternum.

2 months after discharge gained 11 kilos and had no symptoms of cardiac insufficiency. No murmurs heard. Letter of Mar. 28, 1922, reported improvement, and return to work as housemaid. The only symptoms were occasional attacks of palpitation.

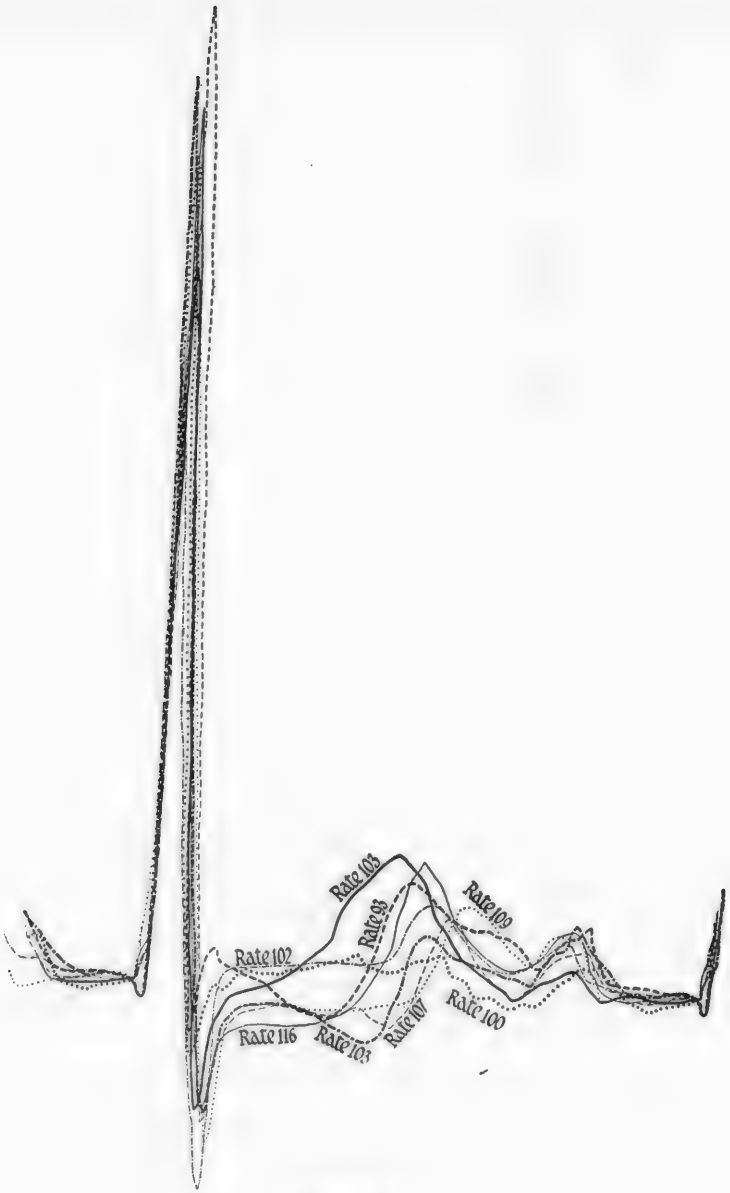


TEXT-FIG. 7. In this figure are reproduced outline drawings of Lead III of 5 electrocardiograms taken from the same patient. Each complex was projected on a screen, the magnification being so arranged that the points of onset either of the Q or R waves of two succeeding cycles were made to coincide. The outline was then drawn. In this way the proportion of the cycle taken by each event is accurately given. The differences in outline, especially in the region of the T waves in the various curves are strikingly exhibited. The influence of the rate of the heart on the relative diastolic time occupied by the T waves is also shown.

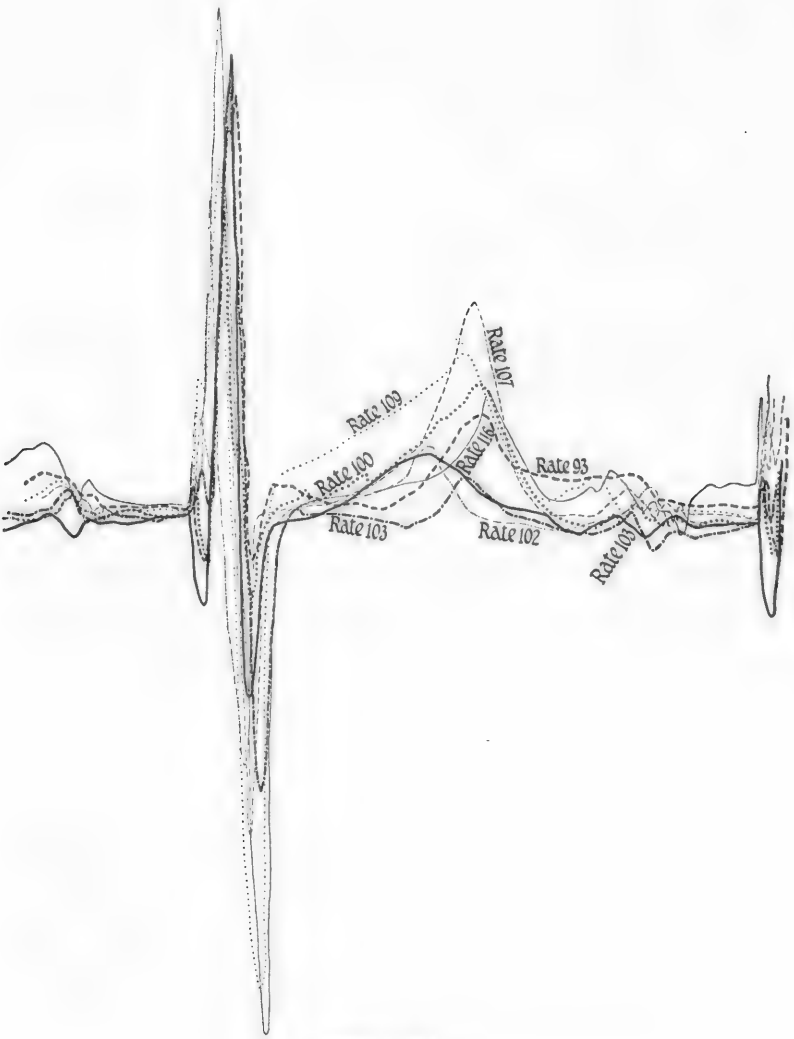


TEXT-FIG. 8, *a*.

TEXT-FIG. 8, *a*, *b*, *c*. Curves constructed in a manner identical with those in Text-fig. 7 are shown. In *a*, drawings of 8 cycles of Lead I are reproduced; in *b*, 8 corresponding cycles in Lead II; in *c*, 8 corresponding cycles in Lead III. The wide variations in form and the influence of rate on form are shown. The change in the level and direction of the curves in the R–T period should be noticed.


Rate 105
Rate 98
Rate 109
Rate 102
Rate 107
Rate 100
Rate 116
Rate 103

TEXT-FIG. 8, *b*.


Rate 107
Rate 109
Rate 100
Rate 106
Rate 93
Rate 103
Rate 102
Rate 105

TEXT-FIG. 8, *c*.

TEXT-FIG. 9. Cycles in Lead III of 4 electrocardiograms, constructed like those in Text-Fig. 7 are exhibited. The R–T period is seen to be little affected. The chief changes are seen in the T wave itself.

EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. A series is shown of electrocardiograms taken of a single patient (J. L., Case 26). In this and the succeeding electrocardiograms the ordinates are 0.04 apart; the abscissæ, 10^{-4} volts. The mechanism is normal except in the curves of Apr. 3 and May 2; in these two, it may be normal, the P wave being isoelectric or, on the assumption that the P wave cannot be distinguished from the R wave, either the auriculoventricular interval is much prolonged, or nodal rhythm is present. An explanation of the form of the curve of June 2, is given in the text. Changes in the form of the T wave are seen in Lead III.

PLATE 2.

FIG. 2. A series is shown of electrocardiograms taken of a single patient (M. G., Case 12). The mechanism is normal in the curves taken on Jan. 27, 31, Mar. 8, 23, and June 23. Fibrillation of the auricles is seen in those of Feb. 16, 21, Mar. 2, and 7. The curve of Mar. 7, Lead II, exhibits a transition from the fibrillatory to the normal mechanism. In the curve of Mar. 6, flutter of the auricles is seen. Ventricular premature beats are seen in the curve of Mar. 2. Prolonged conduction and varying degrees of block occur in the curves of Mar. 8, Apr. 2, 23, 26, and May 11. Curves of Jan. 31 and Feb. 16 show an alteration in the down stroke of the R wave in Lead I, described in the text.

PLATE 3.

FIG. 3. A series of electrocardiograms is shown of a single patient (H. E., Case 30). The curves of Nov. 24, 26, and Dec. 1 show striking alterations in the T waves in Lead I. These may be compared with the second and third curves in Fig. 2. In the curves of Dec. 1, and Mar. 3, premature contractions of ventricular origin are shown.

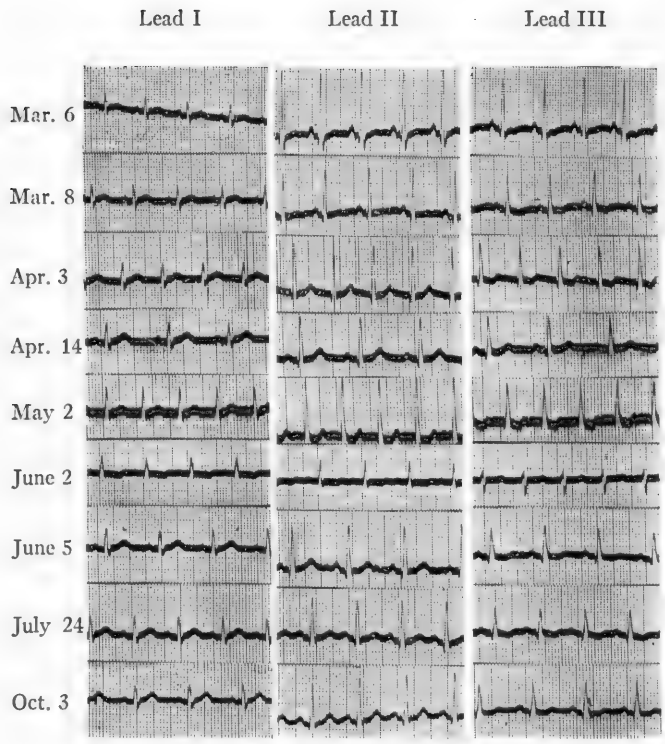


FIG. 1.

(Cohn and Swift: Myocardial involvement in rheumatic fever.)

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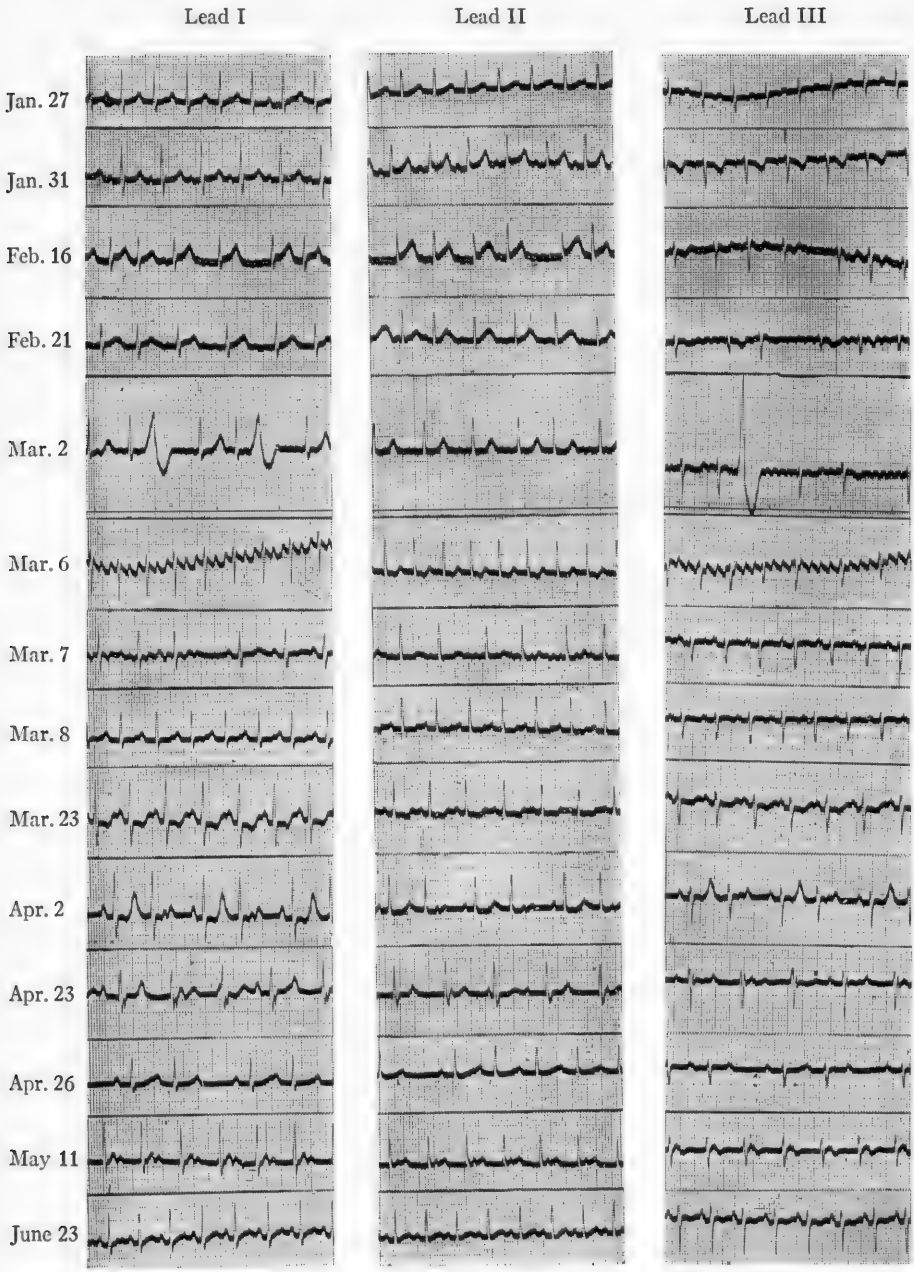

FIG. 2.

(Cohn and Swift: Myocardial involvement in rheumatic fever.)

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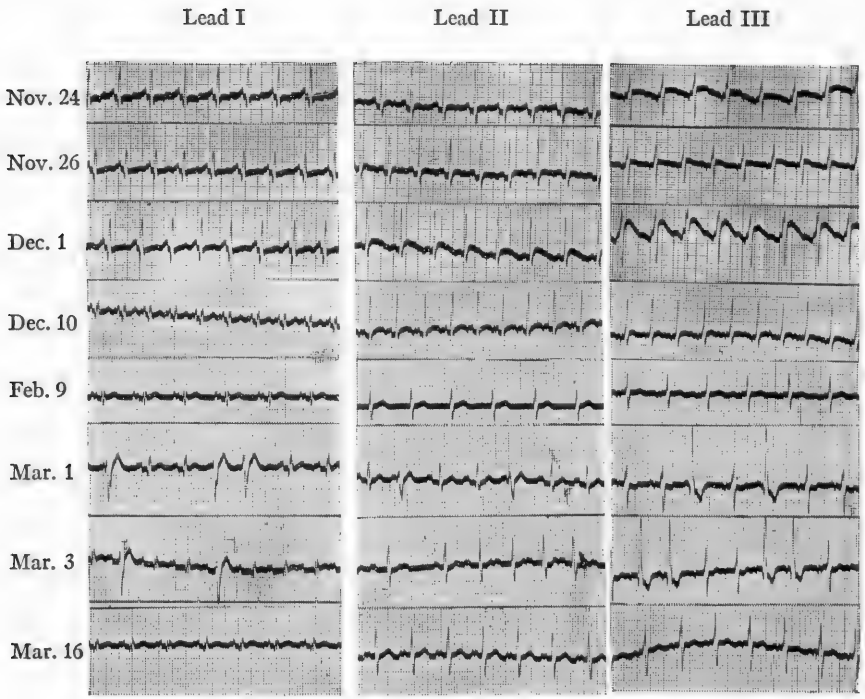

FIG. 3.

(Cohn and Swift: Myocardial involvement in rheumatic fever.)

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SURFACE TENSION OF SERUM.

VII. Significance of the Maximum Time-Drop of Serum Solutions.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 4, 1923.)

In a previous series of papers,[1] we showed that when solutions of rabbit serum are allowed to stand for 2 hours, the value of their surface tension decreases. The difference in dynes between the two values at the beginning and at the end of the 2 hours was termed time-drop. It was observed, moreover, that the presence of antibodies increases the time-drop of a serum.[2]

When different dilutions are made, a maximum drop is observed at 1:10,000.[3] In order to account for this maximum, the view was advanced that, at a given concentration, the number of surface-active molecules, or micellæ, capable of wandering from the bulk to the surface was exactly sufficient to cover the area offered to them, without piling up; that, moreover, these micellæ were organized geometrically side by side, as a pavement, and oriented in the same way, thus forming a homogeneous film limited toward the air surface by one kind of atoms, and toward the liquid surface by another kind. In the present communication, we propose to discuss the significance of the figure 1:10,000, and to deduce the consequences of the above mentioned conception.

Let us assume that this hypothesis is well founded, and that the size and shape of the molecules are invariable. Then, the maximum time-drop in 2 hours is function of the ratio of two quantities,

[1] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 575, 707; xxxvi, 547.

[2] du Noüy, P. L., *J. Exp. Med.*, 1923, xxxvii, 659.

[3] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 707.

volume of liquid and area of this volume; in other words, the maximum drop at 1:10,000 characterizes the vessels used in our experiments. The watch-glasses employed were always filled with 2 cc. of liquid which spread over a free area approximately 12 sq. cm., with a maximum depth of about 2.5 mm. Should the same amount of liquid be allowed to spread over an area twice as great, it is quite obvious that the film could not be formed, for lack of molecules. It would then be necessary to increase their number in the solution; that is, to use a more concentrated solution. In this case, the concentration should be twice as great. Thus, the concentration has to be increased every time the surface is increased with respect to the volume of liquid. In our watch-glasses, assuming that the molecules are adsorbed not only at the free surface but also on the glass, the ratio, $\frac{24 \text{ sq. cm.}}{2 \text{ cc.}} = 12$, corresponds to a homogeneous monomolecular film at a concentration of about 10^{-4}. If this ratio were 120, the film, to be perfect, would require a concentration of 10^{-3} (ten times more molecules per unit of volume), and so on.

It is well known that when a solid, a sphere for instance, decreases in size, the ratio, $\frac{\text{Surface}}{\text{Volume}}$ increases very rapidly. In the case of colloidal particles, the ratio becomes enormous. Consequently, an attempt was made to calculate the dimensions of a container in which the ratio $\frac{S}{V}$ would be so large that the surface-active molecules of pure serum would be adsorbed in an oriented, monomolecular film; in other words, a vessel in which this ratio would be 10,000 times larger than in a watch-glass; *viz.*, 120,000. It was found that, provided the shape (flat disc) is unchanged, it would require a diameter of 5μ (0.0005 cm.) and a maximum thickness of 0.3μ. Though the thickness is almost ten times less, the diameter happens to be of the order of magnitude of the living cells (5 to 15μ). This observation led us to look at the problem from a different point of view, and no longer to consider the liquid contained in the cells, but the surrounding liquid; namely, the plasma in the arteries, and especially in the capillaries. Assuming that in small capillaries, the cells al-

most obstruct the vessels, which is the case as shown by microscopic examination of transparent flaps in live frogs, the calculation was made for a capillary 10μ in diameter in which 2 cells, 5μ in radius and 2μ in thickness, would flow every 10μ. The ratio $\frac{S}{V}$ was equal to 19,300, when the total area and the total volume were taken into account. But capillary vessels are rarely circular in shape; observation shows that the cells often are squeezed and must alter their form in order to pass. Then, assuming a moderate flattening of the vessel, and reducing its section to an ellipse of ratio $\frac{1}{2}$ (great axis $= 10\mu$, smaller axis $= 5\mu$), we find $\frac{S}{V} = 116{,}000$, which is very close to 120,000. Of course, these conditions may be considered as somewhat arbitrary, and it is not our aim to lay emphasis on the quantitative agreement of these figures. However, very little is known concerning the concentration of red and white cells in the capillaries. It varies according to circumstances, probably within great limits. Danzer and Hooker[4] found that one of the annoyances in determinations of the capillary blood pressure in man, when the criterion employed was the cessation of corpuscular flow, lay in the fact that often the large and conspicuous capillaries were filled with stagnated corpuscles, and they noted that in the course of the observations it was not uncommon to find that a previously stagnated vessel had become patent and that, on the other hand, patent vessels became stagnated. In a recent contribution, Krogh[5] reached the conclusion that in muscle at rest many capillary channels are occluded. Moreover, it must be born in mind that the caliber of capillaries is constantly changing and that the changes are independent of the pressure of the blood supplied to them, as was observed as early as 1879.[6] The variations in the diameter of capillaries at rest and during stimulation have been reported by Steinach and Kahn.[7] They observed an

[4] Danzer, C. S., and Hooker, D. R., *Am. J. Physiol.*, 1920, lii, 136.
[5] Krogh, A., *J. Physiol.*, 1918–19, lii, 457.
[6] Roy, C. S., and Brown, J. G., *J. Physiol.*, 1879–80, ii, 323.
[7] Steinach, E., and Kahn, R. H., *Arch. ges. Physiol.*, 1903, xcvii, 105.

average decrease in diameter of from 18.5 to 7.8μ.[8] Interpreting these values as Hooker[9] does in terms of sectional area, the cross-section of the relaxed capillary is found to be 0.0011 sq. mm. as compared with 0.0002 sq. mm. for the contracted capillary, a fivefold difference. Capillaries under 10μ are not so frequently observed to contract; nevertheless, Steinach and Kahn[7] noticed vessels of 10μ reduced to 3 to 4μ in diameter under stimulation. However, conditions are quite different when pure serum circulates in capillaries and when it stands diluted in a watch-glass. The orientation and consequently the space occupied by the adsorbed molecules are probably function of the nature of the boundary, and different in the case of air, glass, and endothelial cells.

For these reasons, no accurate coincidence of figures can be expected and, if a satisfactory accord is found, it must not be looked upon as a reliable expression of the facts, but simply as an indication concerning the order of magnitude. Therefore, it may be stated that if it be assumed that the maximum time-drop in the surface tension of a serum solution is due to the organization in an oriented monomolecular layer of certain active constituents of the serum, it is probable that such a layer corresponds to the optimum

[8] The measurements of capillaries at rest and during stimulation reported by Steinach and Kahn[7] are as follows:

Observation.	Diameter in relaxation.	Diameter in contraction.	Capillary lumen.	Material observed
	μ	μ		
1	26.0	12.0	Still patent.	Nictitating membrane.
2	24.0	7.0	Invisible.	" "
3	23.0	15.0	Narrowed.	Periesophageal "
4	22.0	6.0	Much narrowed.	Nictitating "
5	21.0	4.0	Invisible.	" "
6	19.0	3.0	"	" "
7	18.0	8.0	Narrowed.	" "
8	18.0	10.0	Invisible.	" "
9	18.0	14.0	Notched.	" "
10	14.0	6.0	Invisible.	" "
11	10.5	3.0	"	" "
12	8.4	6.0	Much narrowed.	" "

[9] Hooker, D. R., *Physiol. Rev.*, 1921, i, 112.

activity of these molecules, as there is no piling up or overlapping. Consequently, it is logical to think that in an animal the concentration of the body fluids will be such as to permit their optimum activity. When calculations are made on these assumptions, starting with the size of the capillaries filled with pure blood, it is found that the maximum drop of surface tension, in vessels such as those used in our experiments, must occur at a dilution comprised between 1:3,000 and 1:10,000, which is in conformity with the experiments. Considering the extreme difference of conditions and the necessary inaccuracy of the calculations, this accord is rather striking. It must also be noticed that, should the figures be correct, it would indicate that the pure serum is slightly too concentrated for a perfect distribution of single molecules. This might be expected, as the liquid is in motion instead of at rest. Doubtless, molecules adsorbed at liquid-solid boundaries (such as blood walls of cells and capillaries) are kept in place by much stronger forces than those which are only due to the surface energy of a gas-liquid system.

Thus, it becomes possible to suppose that, in the circulation of the blood and the lymph, the walls of the vessels and all the cells are coated with a polarized layer of active molecules. Conceivably, the wall of the cell itself is not the only membrane which separates the inside from the surrounding fluid; a protective, chemically active film envelops and follows it everywhere. The inner layer of identical and symmetrically disposed atoms, directed toward the cell, differs from the outer layer of atoms, and a slight change in the outside fluid, affecting this outside layer of atoms, may affect in the same way all the atoms of the inside layer which, in turn, will affect the wall of the cell.

This hypothesis is supported by experiments on the time-drop, would give a logical basis to the normal concentration of colloids in the serum, and, further, would explain the powerful action *in vivo* of exceedingly small quantities of substances. It is in accord with the laws of the "best possible utilization" of materials in a living body. The probabilities incline one to suppose that in such highly complicated mechanisms as organized animals which, from a thermodynamical standpoint, are as near perfection as possible, the capital problem of interchanges between body fluids and cells is solved with the utmost efficiency.

AN ATTEMPT TO ISOLATE A FILTER-PASSING VIRUS IN EPIDEMIC INFLUENZA.

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(Received for publication, July 27, 1923.)

In view of the extensive investigations carried on by Olitsky and Gates[1–12] with reference to the causative agent in epidemic influenza, and particularly in view of the fact that the claims of these observers regarding the demonstration in material and cultures from influenzal patients of a filter-passing microorganism have been more or less confirmed by the reports of Gordon[13] in England, and Lister[14] in South Africa, it seemed to us that the epidemic experienced in Toronto during the past winter should not be allowed to pass without an attempt being made to duplicate some of the experiments of these workers.

Attempts to culture a filterable virus were made in this laboratory by Maitland, Cowan, and Detweiler[15] during the epidemic in the winter of 1920. These were unsuccessful, but the work as far as cultures were concerned, was admittedly not exhaustive. The ani-

[1] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.
[2] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 361.
[3] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 713.
[4] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiv, 1.
[5] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 1.
[6] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 553.
[7] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 813.
[8] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxvi, 501.
[9] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxvi, 685.
[10] Olitsky, P. K., and Gates, F. L., *J. Am. Med. Assn.*, 1920, lxxiv, 1497.
[11] Olitsky, P. K., and Gates, F. L., *J. Am. Med. Assn.*, 1921, lxxvi, 640.
[12] Olitsky, P. K., and Gates, F. L., *J. Am. Med. Assn.*, 1922, lxxviii, 1020.
[13] Gordon, M. H., *J. Roy. Army Med. Corps*, 1922, xxxix, 1.
[14] Lister, S., *South African Med. Rec.*, 1922, xx, 434.
[15] Maitland, H. B., Cowan, M. L., and Detweiler, H. K., *Brit. J. Exp. Path.*, 1920, i, 263.

mal experiments, on the other hand, were extensive, but here again we were unable to find lesions which could legitimately be called influenzal in origin. Observations made while this work was in progress led to further experiments, which showed[16] that the lesions which are commonly ascribed to experimental influenza in the literature are in reality due to the method of killing the animal. This view is supported by Lister[14] and has been confirmed during the course of the work on which this paper is based.

The clinical material used was obtained from cases seen by us during the epidemic of influenza occurring in Toronto during the months of January and February of this year. The symptoms and signs considered diagnostic of the disease were abrupt onset with chills or chilly feeling, fever, general malaise with prostration, headache, pains in the back and limbs, suffusion of the eyes and flushed face, and usually a tracheal cough with other signs of upper respiratory affection. Physical examination in the early stages revealed nothing but the signs enumerated above, and the subsequent history of the cases was entirely in keeping with the ordinary course of influenza. While this epidemic, taken as a whole, was of a mild nature compared with that of 1918, there were numerous cases showing all the violence of the first great wave of the disease. Case 6, to be described, was an example of the worst type with fatal lung involvement. Cases with a vague onset were not considered suitable for the purposes of this investigation owing to the importance placed by Olitsky and Gates upon obtaining nasopharyngeal washings during the early hours of the disease. This restriction, coupled with the fact that most of the cases admitted to hospital were past the first 36 hours of the disease, rendered the series very small. We feel, however, that the findings should be reported, especially since, in the opinion of many bacteriologists, the evidence bearing upon this subject is still too meager to form a basis for a conclusive verdict.

Technique.

The technique adopted was, when possible, that described by Olitsky and Gates.[1–3] Briefly it was as follows:

[16] Maitland, H. B., Cowan, M. L., and Detweiler, H. K., *Brit. J. Exp. Path.*, 1921, ii, 8.

1. Collection and Preparation of Material.—Nasopharyngeal washings were obtained by washing the nasopharynx with 50 cc. of sterile saline. Unfiltered washings were cultured aerobically on blood agar and chocolate agar and were in most instances also used for animal inoculations. The remainder of the washings was passed first through a Büchner filter and finally through a Berkefeld V filter.

Lung tissue was ground up with sterile sand and saline in a sterile mortar and passed first through a Büchner filter and then through a Berkefeld V filter.

2. Animal Experiments.—All animals were inoculated intratracheally by the tracheotomy method.[17] Guinea pigs and rabbits were used and the amounts of inoculum varied from 1 to 3 cc., animals being injected with both unfiltered and filtered material. After 24 to 48 hours inoculated animals were killed by a blow on the back of the neck, and the lungs removed with aseptic precautions and examined macroscopically. Pieces of lung were kept for culture aerobically and also for culture in Smith-Noguchi medium. Pieces of lung were also kept for sections. The lung remnants were ground up and filtered as already described. Experience with the method of killing animals just described convinced us that it was almost impossible to avoid accidental hemorrhage and aspiration with the production of petechial or massive hemorrhages in the lungs. For this reason the following method of killing animals was used in the later experiments. The anterior thoracic wall and the heart were cut into with a single snip of the scissors. The chest was opened more widely and the diaphragm incised. The lungs were removed and examined without delay.

3. Methods of Cultivation.—Lung tissue was cultured aerobically in dextrose ascitic broth and on blood agar, and anaerobically in Smith-Noguchi medium. Anaerobic cultivation of lung tissue was soon abandoned because the presence of ordinary organisms made the determination of the existence of *Bacterium pneumosintes* difficult. Filtrates of lung tissue or nasopharyngeal washings were cultured in the following ways.

(*a*) On blood agar aerobically.

(*b*) In dextrose broth aerobically.

(*c*) On blood agar anaerobically. Inoculated blood agar plates were placed in anaerobic jars (McIntosh and Fildes[18] bomb type) and incubated for 8 to 10 days. The jars fulfilled satisfactorily the conditions of complete anaerobiosis.

(*d*) In Smith-Noguchi medium. The reaction of the ascitic fluid used for this medium was pH 8.1. This was slightly more alkaline than the optimum reaction mentioned by Olitsky and Gates. However, experience with a known strain of *Bacterium pneumosintes* (C_{34} Rockefeller tenth generation) which Dr. Olitsky kindly sent for comparison with our strains, convinced us that the conditions for growth of *Bacterium pneumosintes* in our medium were not far from optimal.

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[17] All operations were performed under ether anesthesia.

[18] McIntosh, J., *Med. Research Com., National Health Insurance, Special Rep. Series, No. 12*, London, 1917, 5. Fildes, P., *Med. Research Com., National Health Insurance, Special Rep. Series, No. 12*, London, 1917, 59.

Subcultures were made from this strain in Smith-Noguchi mediums prepared with the unaltered ascitic fluid, and also prepared with the ascitic fluid altered to a reaction of pH 7.8. As the mediums had been prepared for 3 or 4 days, anaerobic conditions were already established when the subculture was made, and growth in each tube was extremely heavy in 72 hours. The only difference detected in films was that the organisms showed more azure staining in the tube prepared with the fluid of reaction pH 7.8.

(*e*) Blood dextrose broth under a vaseline seal was used occasionally. Growth in this medium with Strain C_{34} was apparent in 9 days.

(*f*) *B. coli* broth under a vaseline seal was used only in cultivation of Strain C_{34}. In this medium growth was apparent in 10 days.

Smith-Noguchi medium was preeminently the most satisfactory. The slow sparse growth of Strain C_{34} in blood dextrose broth under a vaseline seal, and in *B. coli* broth under a vaseline seal, when compared with the rapid, vigorous and luxuriant growth of the same strain and generation in Smith-Noguchi medium leaves no room for doubt that this medium should be employed always for primary isolations of organisms of this group. Anaerobic blood agar cultures were found disappointing. We could not satisfy ourselves of the presence of surface colonies on this medium. It is true that *Bacterium pneumosintes* could be seen in films prepared by scraping the surface of the inoculated agar after incubation for 10 days, but the organisms were not sufficiently numerous to prove that growth had occurred. In this our findings were similar to those of Lister.[14]

4. Staining.—Well ripened methylene blue proved so satisfactory that no other stains were used. Our own strains and Strain C_{34} were stained clearly not only with steaming methylene blue, but also with methylene blue in the cold. Films stained in the latter manner were only slightly less clear-cut than those stained by steaming.

Sources of Material.

Case 1.—Nasopharyngeal washings from a patient who developed a typical attack of influenza while in the hospital wards. Washings were taken 24 hours after the onset. Aerobic cultures of the washings showed a pure culture of *Streptococcus hæmolyticus β.*

Case 2.—Nasopharyngeal washings from a patient admitted to the wards with clinical uncomplicated influenza. Washings were taken 72 hours after the onset. Aerobic cultures of the washings showed *Streptococcus hæmolyticus β* and *Staphylococcus aureus.*

Case 3.—Nasopharyngeal washings from a patient admitted to the wards with clinical uncomplicated influenza. Gradual onset 72 hours before admission; acute exacerbation with chills 36 hours before the washings showed *Micrococcus catarrhalis* and *Streptococcus hæmolyticus α.*

Case 4.—Nasopharyngeal washings from our laboratory technician who developed uncomplicated influenza. Washings taken within 12 hours of onset. Aerobic cultures of the washings showed *Micrococcus catarrhalis* and an occasional colony of *Streptococcus hæmolyticus β.*

Case 5.—Nasopharyngeal washings from a medical student who developed uncomplicated influenza. Washings taken within 24 hours of onset. Aerobic cultures of the washings showed *Micrococcus catarrhalis* and an occasional colony of *Streptococcus hæmolyticus β.*

Case 6.—Lung from a patient who died of influenzal bronchopneumonia. Duration of pneumonia 4 days. Clinical picture typical. Lungs post mortem showed the typical lesions of influenzal bronchopneumonia identical with the worst cases seen in the epidemic of 1918. Aerobic cultures of lung exudate showed *Staphylococcus aureus* and pneumococcus.

Results of Animal Inoculations.

In the animals inoculated with unfiltered nasopharyngeal washings the findings in the lungs, both in the gross and microscopically, were those of a diffuse bronchopneumonia. Sections showed moderate edema and congestion and focal areas of polymorphonuclear and lymphocytic infiltration, occasionally filling the alveolar spaces and the lumen of the bronchi. This picture never occurred in animals inoculated with filtered material. Hemorrhages without inflammatory reaction occurred in animals killed by a blow on the back of the neck, but were uniformly absent in those in which the lungs were removed without trauma. This was in accord with previous findings in this laboratory. Apart from the accidental hemorrhage, and apart from the presence in a few instances of the spontaneous chronic pneumonic lesion commonly found[19] in rabbits and guinea pigs, there were no abnormalities in any of the animals inoculated with filtered material.

Results of Cultural Experiments.

Case 1.—Cultures of the original filtrate and of the filtrate from the lungs of animals inoculated with the original material were in all instances sterile.

[19] Smith, T., *J. Med. Research*, 1913, xxix, 291.

Case 2.—Cultures of the original filtrate and of the filtrate from the lungs of animals inoculated with the unfiltered material were in all instances sterile.

Case 3.—The Smith-Noguchi culture of the original filtrate showed after 14 days the presence of definite minute coccobacilli in groups. These we considered morphologically indistinguishable from *Bacterium pneumosintes* described by Olitsky and Gates. Subcultures in Smith-Noguchi medium failed to grow. Cultures from the lung filtrates of animals inoculated with the original material were sterile.

Case 4.—The Smith-Noguchi culture of the filtrate from the lungs of Guinea Pig 1 inoculated with the original filtrate showed after 3 weeks a diffuse growth of very fine coccobacilli morphologically similar to *Bacterium pneumosintes*. Subcultures in Smith-Noguchi medium failed to grow.

The Smith-Noguchi culture of the original filtrate was contaminated. This was not filtered as no suspicious organisms were seen.

Case 5.—The Smith-Noguchi culture of the filtrate from the lung of Guinea Pig 2 inoculated with the original unfiltered material, showed after 3 weeks a diffuse growth of very fine coccobacilli. Subcultures in Smith-Noguchi medium failed to grow. The Smith-Noguchi culture of the original filtrate was contaminated.

Case 6.—Cultures of filtrates of the direct material were in all instances sterile.

The Smith-Noguchi culture of the lung tissue from Guinea Pig 3, which had been inoculated with the original macerated lung tissue, showed 5 days after inoculation numerous very fine coccobacillary forms which we considered morphologically indistinguishable from *Bacterium pneumosintes*. This culture was, however, not pure, but contained also a Gram-negative bacillus. On filtration of this culture the fine coccobacillus was not recovered.

DISCUSSION.

It will be noted that in four (Nos. 3, 4, 5, and 6) out of six cases organisms morphologically similar to *Bacterium pneumosintes* appeared in the Smith-Noguchi medium. In Case 6 the Smith-Noguchi medium containing a piece of suspected lung showed minute coccobacilli, but Gram-negative bacilli were also present. On this

account this case should be ruled out of the group in which suspicious organisms appeared. The reason for the restriction is that it is possible that these minute coccobacilli were merely pleomorphic involution forms of the aerobic Gram-negative bacillus which was present. An aerobic Gram-negative bacillus which grows with difficulty under anaerobic conditions has been encountered in the present study; when this organism is inoculated in small amounts into Smith-Noguchi medium an early (48 hours) diffuse growth of minute bacilli occurs. Morphologically the bacillus growing under these conditions might be confused with *Bacterium pneumosintes*.

The remaining three cultures which it is justifiable to consider suspicious were taken from Cases 3, 4, and 5. Washings were obtained from Case 3, 36 hours after the acute onset, and from Cases 4 and 5 within 24 hours of onset. These were, with the exception of Case 2, the patients which furnished the most favorable material for positive results.

The three suspicious cultures were all obtained in Smith-Noguchi medium and developed slowly (2 to 3 weeks). Subcultures on aerobic medium and in Smith-Noguchi medium remained sterile. The fact that these strains developed slowly and were not viable when subcultured to fresh Smith-Noguchi medium suggested the possibility that the ascitic fluid might be at fault. However, it was found that Rockefeller Strain C_{34} grew luxuriantly in the medium.

The morphology of the three strains we considered similar to that of *Bacterium pneumosintes*. Dr. Olitsky kindly offered to examine these strains for us with a view to identification; accordingly the cultures were forwarded to him. He reported that he considered them morphologically similar to his own strains, but that he had been unable to obtain growth in subcultures, so that they could not be identified with certainty. He also stated that he had frequently lost strains in a similar manner. It may be opportune to refer at this point to Woodcock's[20] contention that the suspicious bodies appearing in these cultures are due to protein precipitate produced by cytolysis of the tissue in the Smith-Noguchi medium. Woodcock's observation was prompted by Gordon's[13] statement that the organism he considered the filter passer of influenza was not bacilloid, but rather coccoid, that it stained with difficulty with methylene blue,

[20] Woodcock, H. M., *J. Roy. Army Med. Corps*, 1922, xxxix, 243.

and that best staining results were obtained with Giemsa's stain. Our suspicious cultures were definitely bacilloid, as was Strain C_{34}. Furthermore, our cultures and Strain C_{34} stained with well ripened methylene blue both by steaming and in the cold. The rapidity of growth of Strain C_{34} in subcultures rules out the possibility of a cytolysin producing this appearance. Moreover, the morphology of these strains is too definite and regular for protein precipitate. During the course of the experiments a great number of smears from sterile Noguchi medium were examined. At no time were any bodies encountered in these films which could be confused with *Bacterium pneumosintes*.

It is possible that the bodies in the cultures which are reported here as suspicious are not identical with the microorganisms reported by Olitsky and Gates. Nevertheless, our own observations and those of Dr. Olitsky indicate that morphologically they are indistinguishable, the only link missing being our inability to obtain successful subcultures. As stated before, this failure has been encountered frequently by Olitsky and Gates, and too definite conclusions should not be drawn from our inability to subculture in three instances.

It is to be hoped that reports of similar investigations will be forthcoming from various centers of research, as it is only by an accumulating mass of evidence that the question of the etiology of epidemic influenza can be settled definitely. Controls should include, in addition to normal individuals, cases of upper respiratory infections of types other than influenza. We hope to be able to study such controls as the opportunity occurs.

SUMMARY.

1. Six cases of epidemic influenza in various stages of disease were investigated.

2. Suspicious cultures of bodies resembling *Bacterium pneumosintes* were obtained in three cases, twice from the lung filtrates of inoculated animals and once from the filtrate of nasopharyngeal washings direct.

3. In these instances material was obtained within 36 hours of the acute onset, thus confirming the observations of Olitsky and Gates.

4. Macroscopic and microscopic findings in the lungs of inoculated animals were uniformly negative.

BRAIN LESIONS OF THE DOMESTIC RABBIT.

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PLATES 4 TO 8.

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In recent years the domestic or laboratory rabbit has come into increasing use for the study of experimentally induced lesions of the meninges and brain. This has been notably the case in connection with the investigations first of epidemic (lethargic) encephalitis and next of febrile herpes. The conflicting results obtained by different investigators and the divergence, sometimes very wide, of their deductions have brought the experimental animal itself under a measure of suspicion. This particular aspect of the subject has been reviewed by Dr. Flexner to whose paper[1] I refer those wishing to pursue the subject in some detail.

It was at Dr. Flexner's suggestion that, along with certain cultivation studies which were being made with the so called viruses of epidemic encephalitis and febrile herpes,[2] I undertook the examination of a large number of rabbit brains for the presence of lesions previously described by Bull[3] and Oliver[4] as existing in these animals before experimental inoculations had been made. The rabbits studied were taken from many sources—the laboratory stock regarded as healthy, others dying of snuffles and affections while under observation preparatory to experimental use, as well as animals which were employed for experimental purposes (tumor transplantation, *Treponema pallidum* inoculation, etc.). The total number of brains taken from these animals was 372.[5] The brains were removed *in*

[1] Flexner, S., *J. Am. Med. Assn.*, 1923, lxxxi, 1688, 1785.

[2] McCartney, J. E., *J. Exp. Med.* (in press).

[3] Bull, C. G., *J. Exp. Med.*, 1917, xxv, 557.

[4] Oliver, J., *J. Infect. Dis.*, 1922, xxx, 91.

[5] I am indebted to Dr. Wade H. Brown and Dr. Louise Pearce for sections of a number of rabbit brains.

toto and then dissected so as to provide portions of the forebrain, midbrain, and medulla. Complete transverse sections were made of the midbrain in many instances, but sections of only selected areas, such as the cortex or basal ganglia, in others. These parts were cut in the usual manner after hardening in either Zenker's fluid or in 10 per cent formalin diluted with normal saline solution. Some of the brains were frozen immediately after removal from the rabbit's body and then sectioned. All the sections were stained with hematoxylin and eosin, but some showing lesions were stained in addition by Giemsa's method. In other words, the brains were dealt with in the manner of specimens coming from animals in which definite experiments had been carried out to induce encephalitis.

Possibly it was this extended and rather complete exploration of the brain which accounts for the high percentage of lesions detected.

Results of Examination.

The lesions found in the brains of these rabbits are characterized chiefly by the presence of infiltration with mononuclear cells and less often by peculiar localized necrosis. The infiltrative changes may be classified, depending on the location, into four types: (*a*) perivascular, (*b*) meningeal, (*c*) cerebral, and (*d*) under the ependyma of the lateral ventricles.

This classification does not separate the lesions into hard and fast groups, as, in given cases, infiltrations in all four sites may occur together with localized necrosis. Usually the perivascular, meningeal, and cerebral types of lesions are found in the same case, but in others one of the types may predominate.

Of the class of infiltrative lesions, the histopathology noted in each of the groups is as follows:

(*a*) *Perivascular Lesion.*—This is the commonest and most striking pathological picture observed. The perivascular infiltration may vary from a single layer of mononuclear cells to a thick, dense sheath which, at times, almost obscures the blood vessel. Often the Virchow-Robin spaces surrounding the vessel may be filled with such cells, which are small, round, and deeply staining (Figs. 1 to 6).

(*b*) *Meningeal Lesion.*—Meningeal involvement is not quite so common as the perivascular, and consists of infiltration into the pia-

arachnoid of mononuclear cells. The cellular reaction is often so marked that there is a broad band of cells on the surface of the brain from which proceed finger-like masses of cells dipping into the brain tissue and surrounding the blood vessels. These changes may occasionally be slight, but definite, and appear as a few cells lying apparently between the pia and the brain surface (Figs. 4, 5, 6, and 16).

(*c*) *Cerebral Lesion.*—Changes in the cerebral substance are more common than those in the meninges, and are only slightly less frequent than the lesions occurring around the vessels. They are usually associated with the latter. There is an infiltration with mononuclear cells which may be diffuse, cells being scattered throughout the tissue. Or, more often, there are focal aggregations of cells distributed irregularly throughout the brain. These foci are sometimes large enough to be seen with the naked eye in the stained specimen. As a rule, the neighboring blood vessels show marked perivascular lesions (Figs. 5, 9, 10, 13, and 16).

(*d*) *Lesion under the Ependyma of the Lateral Ventricles.*—This picture is seen least often. It may be found only in the forebrain. If care is taken in differentiating the mononuclear cells from nerve cells present in this region, a more or less dense layer of the mononuclear cells is noted immediately underneath the ependyma (Figs. 7 and 8).

Lesions with Necrosis.—In 15 per cent of the total number of brains examined, there appeared, in addition to the infiltrative changes, localized necrotic areas of peculiar structure. The center is definitely necrotic and contains granular débris. Surrounding this is a zone of cells, each having a large, faintly staining nucleus with an open chromatin network in which is a well defined nucleolus. The cells in relation to the necrotic center are phagocytic and are filled with deeply staining granular detritus. Around this cellular zone is a layer of varying thickness of mononuclear cells. The adjacent blood vessels show rings of similar cells. Under higher magnification an increase of glial tissue surrounding the lesion may be seen, simulating a capsule. If the section is not cut through the center of the necrotic focus, the inner, larger, epithelial-like cells and the surrounding ring of mononuclear cells only are manifest.

In some sections, smaller foci are observed, while in others, a process of healing with definite scar tissue occurs. Occasionally groups of four or five of the epithelial-like cells, without any mononuclear infiltration, may be noted as small islands in the brain substance (Figs. 11 to 16).

TABLE I.

Distribution and Incidence of Cerebral Lesions.

Source of rabbits.	Marked.	Slight.	Negative.	Total.
Transplantable tumor	63	10	73	146
Per cent	43	7	50	
Total per cent positive	50			
Treponema pallidum inoculation	28	5	49	82
Per cent	34	6	60	
Total per cent positive	40			
Uninoculated stock	23	8	21	52
Per cent	44.5	15.5	40	
Total per cent positive	60			
Miscellaneous diseases	30	5	15	50
Per cent	60	10	30	
Total per cent positive	70			
Snuffles	32	0	10	42
Per cent	76		23	
Total per cent positive	76			
Total	176	28	168	372
" per cent	47.5	7.5	45	
Total per cent positive	55			

Table I shows the distribution and incidence of these lesions and the condition of the rabbit in which they were found. The histopathology is recorded as marked and slight. The marked lesions are easily discernible; the slight ones are definite though less frequent but indicate distinct pathological change.

From this table it is apparent that the lesions of meningoencephalitis were present in more than half (55 per cent) of the 372 rabbits examined, of which none was injected intracerebrally. The incidence of cerebral changes in animals inoculated with a transplantable tumor, or with *Treponema pallidum* materials, and normal rabbits was approximately the same (40 to 60 per cent). In the animals suffering from miscellaneous diseases such as pneumonia, septicemia, and subcutaneous abscesses, the percentage was higher, reaching 70 per cent. In rabbits ill with snuffles, 76 per cent were affected. 47.5 per cent of the 372 rabbits showed marked changes.

Inoculation of Cerebral Tissue, Containing Lesions, into Other Rabbits.

Fifteen young rabbits (800 to 900 gm.) were inoculated intracerebrally, after etherization, with 0.25 to 0.35 cc. of suspensions of ground brain tissue obtained from ten rabbits. These latter revealed the cerebral lesions in frozen sections made just prior to use. In none of the fifteen animals were any symptoms noted. They were killed at periods from 14 to 30 days after inoculation and the brains were sectioned and examined. Three exhibited typical changes in the brain, but no conclusions could be drawn, since uninoculated animals of the same size and weight, used as controls, also showed similar pathological conditions.

Cultivation Tests.

Brains were removed from rabbits under aseptic conditions and frozen sections were made. If lesions were present, cultures were made of the brain in a variety of media consisting of ordinary dextrose broth, blood agar in aerobic and anaerobic plates, and Smith-Noguchi medium. In none of these materials was any visible growth obtained.

DISCUSSION.

It appears that approximately one-half of the 372 rabbits examined show particular lesions in the brain. They consist of mononuclear infiltration, either surrounding the blood vessels, under the ventricular wall, throughout the meninges, and in the cortex, or, more frequently, in more than one of these areas. In addition, peculiar necrotic

structures are present in the cortical brain substance. This percentage is maintained not only in animals which are normal by a complete histological examination, but also in rabbits inoculated with a non-infective, transplantable tumor, and in those in the early stages of experimental syphilis. The proportion of animals showing such lesions is increased in the miscellaneous disease group; in the snuffles series more than three-fourths may exhibit evidences of a meningoencephalitis. It has been shown in another paper[6] that in ordinary stock animals free from nasal discharge, about 25 per cent exhibit chronic sinusitis. On the other hand, snuffles is not the only factor, as animals free from any sinus or mastoid infection and in which no evidence of disease can be found macroscopically may show pronounced cerebral changes. Furthermore, in the disease group, which does not include snuffles but other infections, such as coccidiosis, subcutaneous abscesses, and pneumonia, there is also a higher percentage of brain involvement than in the normal. However, the presence of a transplantable tumor, or early experimental syphilis, does not materially affect the proportion in which these changes are found. Attempts at transmission and cultivation of brain tissue showing these lesions have failed to yield positive results.

The frequent occurrence of accidental lesions of a wide variety in the brain of the laboratory rabbit should have an important bearing on the interpretation of experimental results based on the presence of similar cerebral changes in this animal. This is strongly emphasized by Dr. Flexner,[1] and an examination of the literature supports this view.

For example, Reasoner[7] noted, in experimental syphilis of the rabbit, meningeal and perivascular infiltration with mononuclear cells, associated with focal necrotic areas in the cortex. Indeed, these changes are described as specific for this experimental disease. In Fig. 6 of Reasoner's paper, a meningitis is shown; in Fig. 7 a focal necrotic lesion; and in Fig. 8 a perivascular infiltration labelled "characteristic syphilitic blood vessel." If a comparison be made between these photographs and Figs. 2 to 7, 11 to 14, and 16 of the present article, it appears that normal rabbits, those inoculated with a transplantable tumor, and those suffering from snuffles or other diseases, reveal practically the same pictures.

[6] McCartney, J. E., and Olitsky, P. K., *J. Exp. Med.*, 1923, xxxviii, 591.
[7] Reasoner, M. A., *J. Am. Med. Assn.*, 1916, lxvii, 1799.

Moreover, Bull[3] presents photographs (Figs. 12 to 17) of brains from rabbits which succumbed to bacillary infection or were killed during a streptococcus infection, in which the perivascular infiltration, the meningitis, and the focal necrotic lesions are definitely exhibited. So does Oliver,[4] whose illustrations (Figs. 1 and 2) of meningeal, perivascular, and cerebral necrotic lesions were taken from supposedly healthy rabbits of the laboratory stock. It is therefore apparent that these cerebral changes can hardly be considered as specific manifestations of experimental syphilis in the rabbit.

Recently a number of investigators have reported the experimental production of lesions of the meninges and brain by inoculating rabbits with the so called viruses of epidemic (lethargic) encephalitis and of febrile herpes. Their conflicting results are probably due to a lack of recognition of spontaneous or accidental cerebral lesions in this experimental animal.

For example, Loewe and Strauss[8–13] report that the cerebral changes which consist chiefly of a meningeal and perivascular mononuclear infiltration associated with areas of necrosis surrounded by a zone of mononuclear cells indicate the action of the so called virus of encephalitis lethargica. They obtain similar results with their supposed cultures of this material. They point out that in doubtful cases of the disease in man, the intracerebral inoculation of the patient's spinal fluid in the rabbit results in the same lesions, which suffice for a diagnosis of epidemic encephalitis. These observers are supported by Thalhimer.[14] Now, if a comparison is made of Loewe and Strauss' photographs of the meningitis (Fig. 8 of their article[13]), the perivascular infiltration (Figs. 9, 10, 12, 13, 14, and 15 of the same article, Fig. 4 of another of their papers[11]), the focal infiltration with mononuclear cells (Figs. 11 and 13 of the first article[13]), the focal necrotic areas (Fig. 10 of the same article), together with Figs. 4 to 8 of Thalhimer's series,[14] with Figs. 1 to 16 illustrating the present paper, it will be observed that the same kind of lesions can occur in supposedly healthy animals, or in those employed for a variety of experimental purposes other than for intracerebral inoculation. A further comparison may be made with the photographs of similar conditions in animals free from the so called encephalitic virus presented by Bull[3] and by Oliver[4] and to which reference has already been made.

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[8] Loewe, L., Hirschfeld, S., and Strauss, I., *J. Infect. Dis.*, 1919, xxv, 378.

[9] Loewe, L., and Strauss, I., *J. Am. Med. Assn.*, 1920, lxxiv, 1373.

[10] Loewe, L., and Strauss, I., *J. Am. Med. Assn.*, 1919, lxxiii, 1056.

[11] Loewe, L., and Strauss, I., *J. Infect. Dis.*, 1920, xxvii, 250.

[12] Acute epidemic encephalitis. An investigation by The Association for Research in Nervous and Mental Diseases, New York, 1921.

[13] Loewe, L., and Strauss, I., *Proc. New York Path. Soc.*, 1920, xx, N.S., 18.

[14] Thalhimer, W., *Arch. Neurol. and Psychiat.*, 1922, viii, 286.

Kling and his coworkers[15–20] also emphasize the presence of the cerebral lesions in the rabbit as evidence of the experimental transmission of epidemic encephalitis and of febrile herpes. In describing the histopathology, they state: Epidemic encephalitis is characterized not only by perivascular lesions but also by chronic foci having a necrotic center with epithelial cells and surrounded by lymphocytes.[20] Kling[17] reports that from 50 to 60 per cent of the inoculated rabbits reveal these cerebral changes. On the other hand, of the present series of 372 brains from a supposedly normal, laboratory stock of animals and from those not inoculated intracerebrally or with encephalitic or herpetic materials, 55 per cent show a similar histopathologic condition. This can readily be seen by comparing the photographic reproductions of what Kling and his coworkers regard as specific encephalitic changes as shown in their Figs. 1, 3, 4, 5, 8,[18] Figs. 1, 2, and 4,[19] and in others,[15] with Figs. 1 to 7, 13, and 16 of this article. In the figures of the present article can be seen precisely similar meningeal and perivascular infiltrations, and focal mononuclear lesions. The peculiar necrotic area which they consider as pathognomonic of experimental encephalitis (their Fig. 1[20]), and which consists of a central necrotic area containing epithelial-like cells surrounded by a zone of mononuclear cells, was found in 15 per cent of the rabbits, supposedly normal or not inoculated with encephalitic materials. An identical lesion can be observed in Figs. 11 to 16, presented herewith, and taken from a supposedly normal stock rabbit, from one with snuffles, from three with a transplantable tumor, and from one in the early stages of experimental syphilis.

This study leads to the conclusion that the cerebral lesions found so frequently in the domestic rabbit are preexisting; that is, they are present before any experimental procedure is begun. Many suggestions have been made regarding their origin. Wright and Craighead[21] in 1922 described a protozoon which they consider to be the cause of infectious motor paralysis of young rabbits. This organism in turn agrees in part with the *Encephalitozoon cuniculi* described

[15] Kling, C., Davide, H., and Liljenquist, F., *Compt. rend. Soc. biol.*, 1921, lxxxiv, 815; lxxxv, 823.

[16] Kling, C., *Hygiea, Stockholm*, 1922, lxxxiv, 894, 913.

[17] Kling, C., *Wien. Arch. inn. Med.*, 1923, vi, 101.

[18] Kling, C., Davide, H., and Liljenquist, F., *Hygiea, Stockholm*, 1921, lxxxiii, 705.

[19] Kling, C., Davide, H., and Liljenquist, F., *Hygiea, Stockholm*, 1921, lxxxiii, 566.

[20] Kling, C., Davide, H., and Liljenquist, F., *Compt. rend. Soc. biol.*, 1922, lxxxvii, 1179.

[21] Wright, J. H., and Craighead, E. M., *J. Exp. Med.*, 1922, xxxvi, 135.

by Levaditi, Nicolau, and Schoen,[22] who believe it to be the etiological agent of the particular cerebral lesions under discussion. Before the appearance of the latter papers, however, Dr. Goodpasture kindly lent to The Rockefeller Institute preparations revealing somewhat similar organisms which he also considers as the incitant of the pathological changes.[23] By comparison with these specimens and with the aid of the foregoing published data, Dr. Cowdry[24] has been able to observe the protozoon-like structures in some of the brains described in the present paper. The view that some other factors than the so called virus of epidemic encephalitis or of febrile herpes induce the lesions in the brains of rabbits is also in conformity with the observations of Doerr and Zdansky,[25] and others.[26]

CONCLUSIONS.

Lesions of meningoencephalitis were found in 55 per cent of 372 rabbits comprising the laboratory stock regarded as healthy, others with snuffles or dying from different affections while being kept under observation, and still others which were employed for experimental purposes, such as tumor transplantation and *Treponema pallidum* inoculation. None was injected intracerebrally. The lesions consist in the main of infiltration with mononuclear cells occurring around the blood vessels, in the meninges, in the cortex, and under the ependyma of the lateral ventricles, together with particular focal necrotic areas in the cortex. The incidence of these histopathological changes varies in different series of animals; in those supposedly normal and in rabbits inoculated with a transplantable tumor or with *Treponema pallidum* material, the percentage of positives was from 40 to 60; in those suffering from miscellaneous diseases, such as pneumonia, septicemia, etc., the percentage was 70, and in rabbits ill with snuffles,

[22] Levaditi, C., Nicolau, S., and Schoen, R., *Compt. rend. Acad.*, 1923, clxxvii, 985; *Compt. rend. Soc. biol.*, 1923, lxxxix, 984. Levaditi, C., and Nicolau, S., *Compt. rend. Soc. biol.*, 1923, lxxxix, 775.

[23] Goodpasture, E. W., personal communication.

[24] Cowdry, E. V., personal communication.

[25] Doerr, R., and Zdansky, E., *Schweiz. med. Woch.*, 1923, iv, 349.

[26] Twort, C. C., and Archer, H. E., *Lancet*, 1923, i, 1102.

as many as 76 per cent were affected. Marked lesions were observed in 47.5 per cent of the total.

The histopathological picture observed in these rabbits corresponds to those offered by a number of investigators as evidence of the transmission of certain nervous diseases of man to this animal. The accidental cerebral lesions in the rabbit, of a wide variety, and of frequent occurrence, are to be regarded as existing before any experimental procedure is begun. Their recognition is of the utmost importance in the interpretation of experimental results based on the presence of similar changes in this animal.

EXPLANATION OF PLATES.

PLATE 4.

FIG. 1. Section of the brain of a rabbit injected intratesticularly with *Treponema pallidum* material, showing perivascular infiltration with mononuclear cells in the Virchow-Robin space. × 640.

FIG. 2. Section of the brain of a supposedly normal stock rabbit. To be noted are the numerous perivascular lesions. × 200.

FIG. 3. The same field as Fig. 2 under higher magnification, illustrating nature of the mononuclear cells. × 365.

PLATE 5.

FIG. 4. Section of rabbit brain from an early case of snuffles, showing slight meningeal infiltration with mononuclear cells. There is some perivascular infiltration around the small blood vessel. × 365.

FIG. 5. Section of the brain of a rabbit inoculated intratesticularly with a transplantable tumor, showing meningeal, cerebral, and perivascular infiltration. × 100.

FIG. 6. Section of the brain of a rabbit with snuffles, containing meningeal and perivascular infiltrations. × 250.

PLATE 6.

FIG. 7. Section of the brain of a rabbit with early snuffles, revealing slight mononuclear infiltration under the ependyma of the lateral ventricle. × 210.

FIG. 8. From a similar rabbit, but with more marked infiltration with mononuclear cells under the ependyma of the lateral ventricle. × 250.

FIG. 9. Section of the brain of a rabbit with a subcutaneous abscess. Diffuse infiltration with mononuclear cells into the cerebral substance is seen. × 280.

FIG. 10. Brain of a rabbit inoculated with a transmissible tumor, showing small groups of mononuclear cells in the brain substance. × 250.

PLATE 7.

FIG. 11. Brain from a similar rabbit with a well marked area of focal necrosis. To be noted are the necrotic center, large, epithelial-like cells, and the surrounding mononuclear infiltration. × 190.

FIG. 12. A similar area of necrosis found in a supposedly normal stock rabbit. × 190.

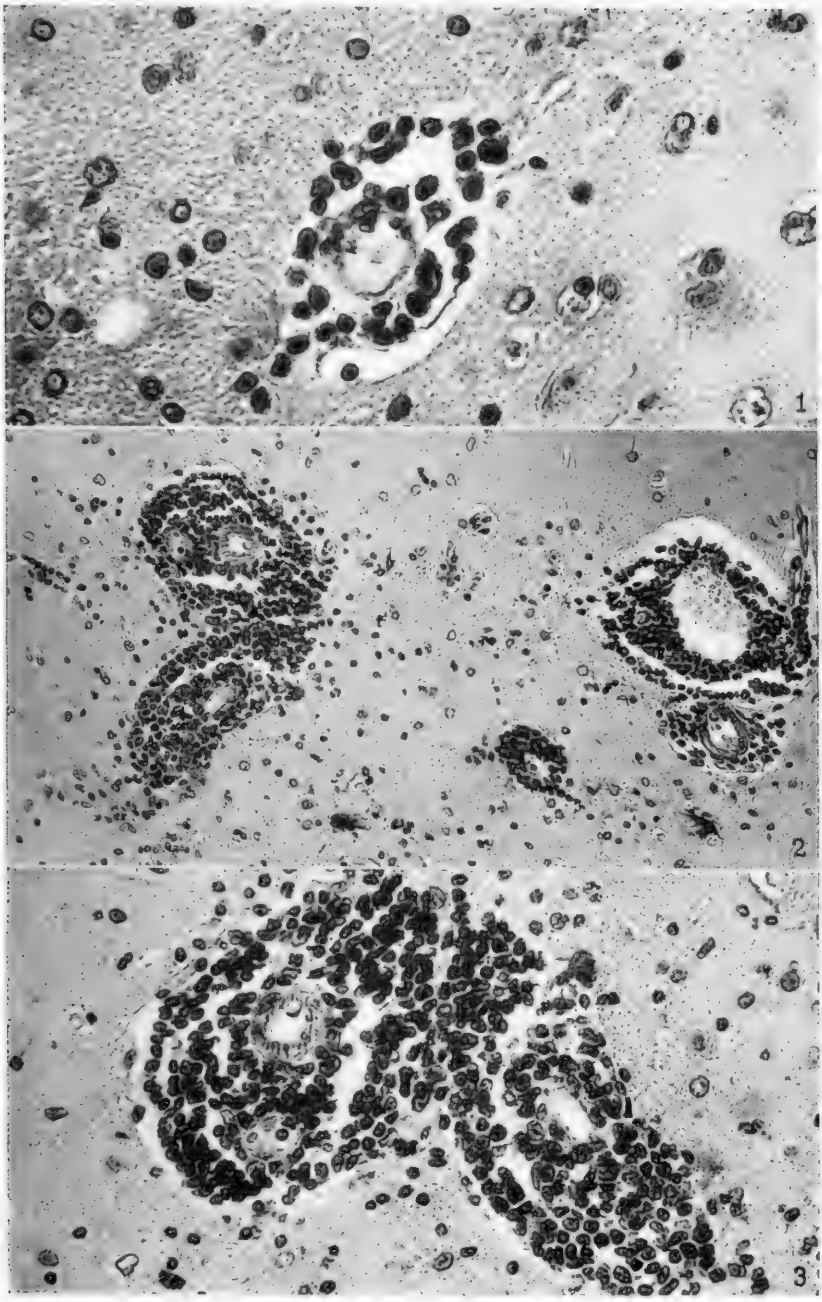
FIG. 13. Section of the brain from a snuffles rabbit, showing a similar area of focal necrosis, but not cut through the center. The blood vessels reveal marked perivascular infiltration. × 190.

PLATE 8.

FIG. 14. Brain from a rabbit inoculated intratesticularly with a transmissible tumor. A focal necrotic area with infiltration by mononuclear cells is seen. × 270.

FIG. 15. Section of the brain of a rabbit injected intratesticularly with *Treponema pallidum* material, showing an area of focal necrosis in process of healing. × 250.

FIG. 16. Section of the brain of a rabbit inoculated intratesticularly with a transmissible tumor. To be noted is an area of necrosis at the surface of the brain with meningeal and perivascular infiltration. × 110.



(McCartney: Brain lesions of domestic rabbit.)

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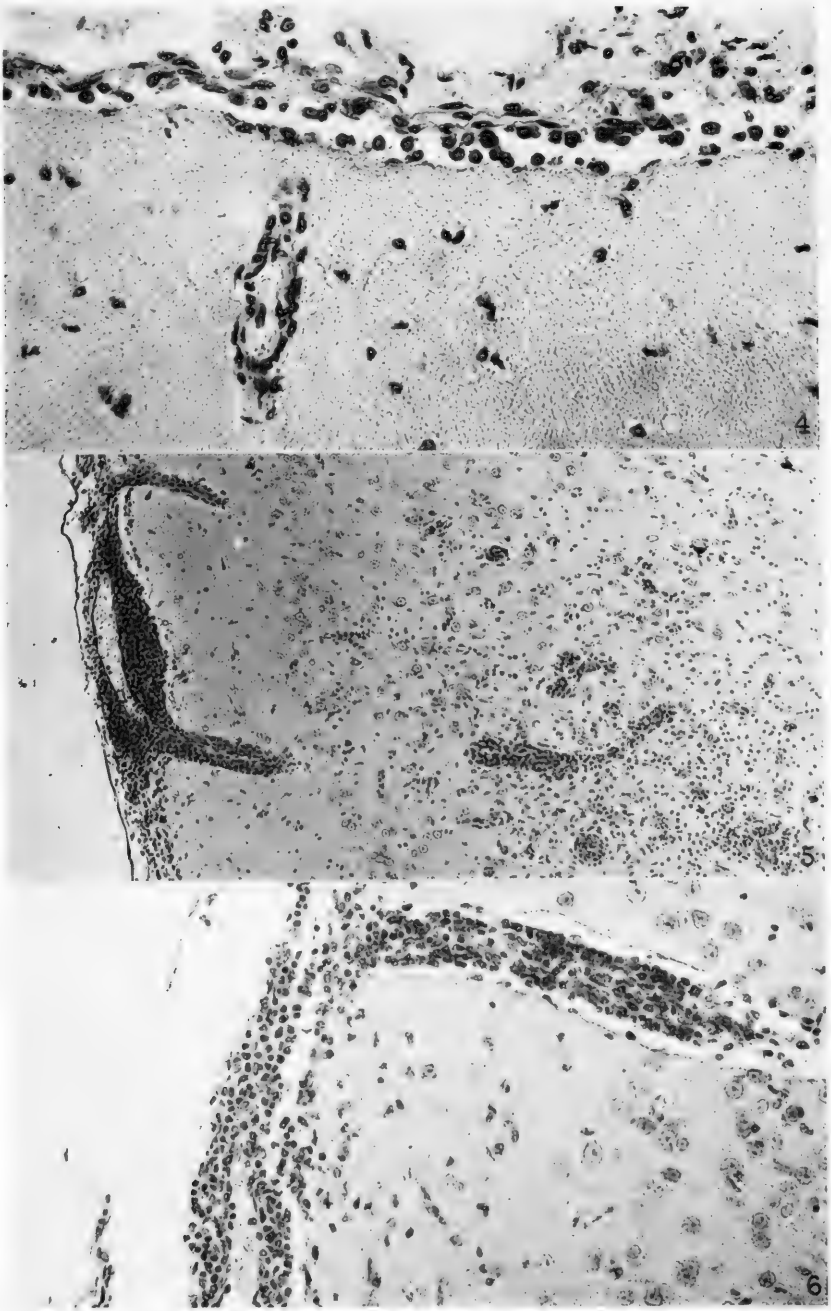

(McCartney: Brain lesions of domestic rabbit.)

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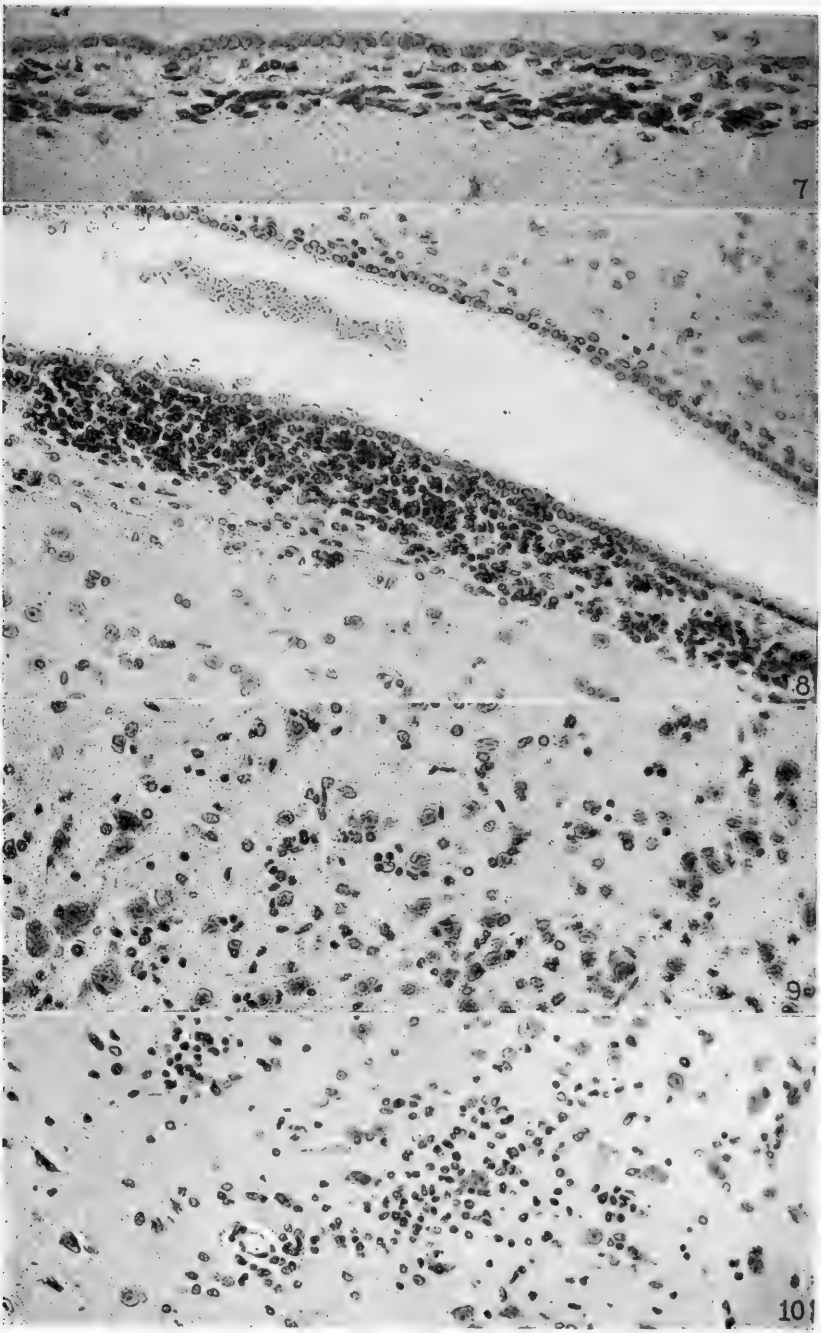

(McCartney: Brain lesions of domestic rabbit.)

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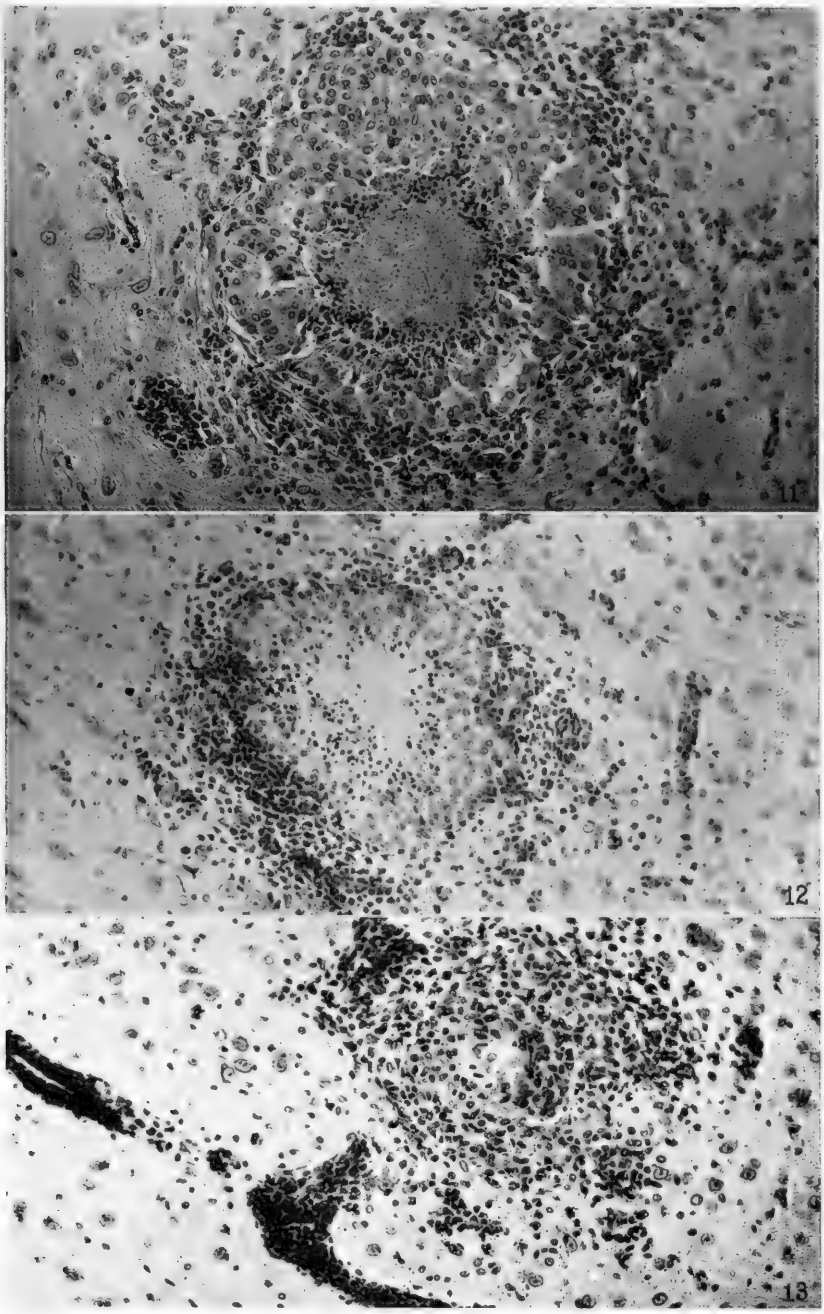

(McCartney: Brain lesions of domestic rabbit.)

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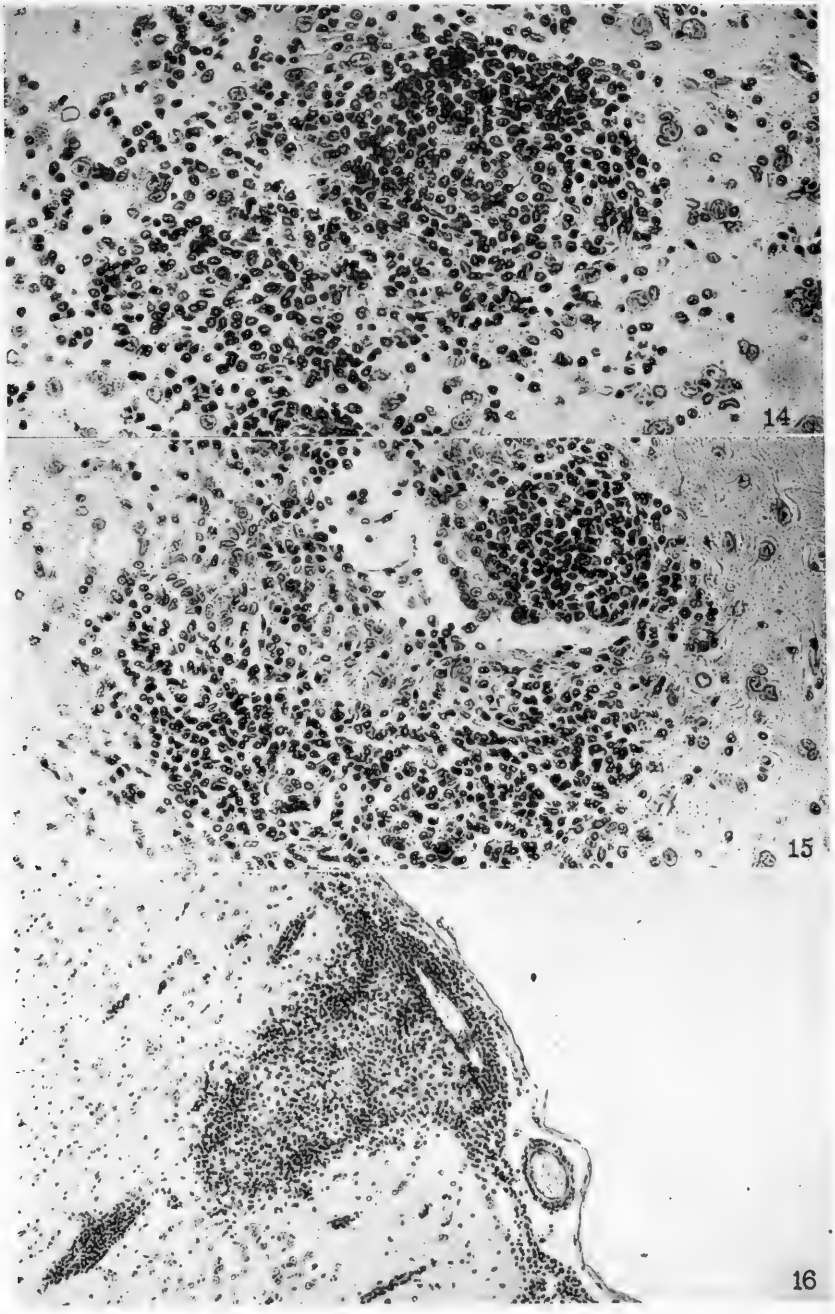

(McCartney: Brain lesions of domestic rabbit.)

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AN EXPERIMENTAL STUDY OF MITOCHONDRIAL CHANGES IN THE THYROID GLAND.

By F. M. NICHOLSON, Ph.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 9 to 11.

(Received for publication, August 1, 1923.)

That the thyroid plays an important, though little understood, part in growth and in the coordination and regulation of vital phenomena has been demonstrated repeatedly—with unusual clearness in the case of amphibia[1]—and it is fully recognized by physiologists. We are also familiar with some of the more conspicuous constitutional disturbances which follow derangements of the normal activity of the gland. We suspect that the latter is associated in some way with the development of important racial characteristics.[2] And only recently evidence has been presented that resistance to certain infections is likewise in some obscure way linked up with the degree of development (or activity) of the thyroid and of other endocrines. This information has come through the systematic study of experimental syphilis and tumor metastases in rabbits by Brown and Pearce[3] carried out upon a sufficiently large scale to minimize individual variations. The authors mentioned found that changes occurred in the size and in the gross and microscopic appearance of the thyroid gland coincidently with definite stages in disease reactions. Unfortunately, any explanation of the basic phenomena is prevented at the outset by our lack of comprehension of the mechanism of thyroid secretion. The gland is constructed upon a peculiar and totally different plan from any others.

[1] Gudernatsch, J. F., *Arch. Entwcklngsmechn. Organ.*, 1912–13, xxxv, 457.
Swingle, W. W., *J. Exp. Zool.*, 1922, xxxvi, 397.
Uhlenhuth, E., *Proc. Soc. Exp. Biol. and Med.*, 1922–23, xx, 494.

[2] Keith, A., *Lancet*, 1919, ii, 553.

[3] Brown, W. H., and Pearce, L., *Proc. Soc. Exp. Biol. and Med.*, 1922–23, xx, 472, 476.

Recognizing the fact that investigators have advanced about as far as can reasonably be expected with data regarding the size and arrangement of the cells and their nuclei and the appearance of the intrafollicular colloid, Goetsch[4] sought for a more delicate criterion of thyroid activity, and made an investigation of the mitochondria in pathologic human thyroids. He was rewarded by considerable success in correlating mitochondrial changes with clinical symptoms—a result which is fully in accord with the large mass of information at hand bearing upon the probable physiological significance of these granulations. In the hope that an analysis of the experimental conditions which lead to definite alterations in the shape and number of mitochondria in the comparatively easily controlled thyroid glands of guinea pigs, might contribute a little to the general problem, the following experiments were undertaken.

EXPERIMENTAL.

Normal Control.

Since the thyroid is notorious for its variability in apparently normal animals, it was first essential to determine the usual appearance of its mitochondria. This necessitated considerable experimentation in technique and the observation of a selected group of guinea pigs held under slightly different physiologic conditions within the limits of normality.

After tests with many fixatives, it was discovered that the shrinkage of the intrafollicular colloid and the resultant distortion of form relations could be avoided by fixation in a mixture devised by Pianese and composed of the following ingredients.

1	per cent	aqueous	solution	chloride of platinum and sodium	15 cc.
¼	"	"	"	chromic acid	5 "
2	"	"	"	osmic acid	5 "
Formic acid c. p.					1 drop.

This fixative was, moreover, found to be an excellent preservative for mitochondria, which, after its use, may be stained easily by treatment with fuchsin and methyl green which gives them a bright crimson color and makes them stand out sharply against a light green background. Because of the tendency of some fluids to

[4] Goetsch, E., *Bull. Johns Hopkins Hosp.*, 1916, xxvii, 129.

cause a partial fragmentation of mitochondria so that they are rendered somewhat more granular than in the living gland, the results were in several instances checked by the Janus green staining of mitochondria in living cells and found to be substantially correct. Other preparations made by the methods of Bensley and Regaud were employed for controls. In estimating the number of mitochondria, care was taken throughout the experiments to secure a uniform degree of differentiation by washing the preparations in water and counterstaining with methyl green to a uniform extent. The mixture of Pianese has the advantage over the fixatives of Altmann and Flemming in that it penetrates easily and rapidly, so that an even preservation of mitochondria throughout the fragment of tissue can be obtained. Short serial sections were usually made in a plane parallel with the long axis of the gland and they were cut at a uniform thickness of 4 microns. The same routine was always followed in fixation and dehydration, embedding, and staining, so the results are strictly comparable. In all cases involving ligation of blood vessels, the control and experimental tissues were fixed in the same bottle, embedded in the same block of paraffin, placed on the same slide, stained together, and mounted under the same cover-glass; but in other experiments this precaution was not considered necessary.

Unfortunately, the guinea pigs were purchased from several dealers and accordingly came from a mixed stock so that their antecedent history and diet were not known. In some cases they were given a short time to adjust themselves to laboratory conditions. Failure to do this always does not vitiate the results for the reason that the control findings remained remarkably constant while changes in the mitochondria did not occur spontaneously but always in response to experimental influences. The animals were kept in individual cages, under similar conditions, and upon a practically constant diet. In consideration of the profound influence of the nervous system upon the thyroid, they were treated with extreme gentleness and unusual care was taken in other ways not to excite them. To procure tissues for examination the animals were killed with chloroform or ether. Many rats and rabbits were employed also for vitamine and insulin experiments, and the normal was carefully standardized in both cases (see Figs. 26 and 37).

A systematic examination was made by this technique of tissues from 13 adults and 11 young normal guinea pigs in various stages of development. The appearance of the mitochondria in the thyroids of normal young adult guinea pigs is represented in Fig. 4. The mitochondria seemed to be identical in the epithelium of large and small follicles, but follicles of about the same size were selected for comparison to make it more direct. The findings remained constant from day to day throughout the period of experimentation (October to June). No mitochondrial differences were detected between the

sexes except during pregnancy. Although the mitochondria were relatively more numerous in embryonic thyroids (Fig. 1) and most abundant after birth (Figs. 2 and 3) their uniformity was remarkable in young adults of about 325 gm. such as were chosen for experimentation (Fig. 4). Old animals, unless very senile and almost moribund, showed no distinctive changes in the mitochondrial content of the thyroid (Fig. 5).

Interference with Blood Supply.

In surgical practice, one of the simplest and most direct methods of reducing thyroid activity is by ligation of the large arteries supplying the gland. To ascertain how the mitochondria respond to this treatment, the larger vessels to one gland were ligated in a series of animals, the gland on the other side being left untouched. Both glands were removed and examined cytologically at the following intervals after operation: 2 animals after 1 day; 3 after 2 days; 2 after 3 days; 3 after 4 and 5 days, respectively; 2 after 6 and 7 days, respectively; 3 after 8 days; 2 after 9 days; and 1 after 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 days.

No alterations were noted after 1 and 2 days of reduced blood supply but after 3 days, the mitochondrial filaments had become shorter and more granular (Fig. 6). This change gradually became more and and more pronounced each succeeding day up to the 10th day, as illustrated in Figs. 7, 8, 9, and 10, when all the mitochondria had rounded up into spherules. That the response was a very delicate one is shown by the fact that no parallel modifications could be detected in the shape of the follicles, in the appearance of the nuclei, or in the condition of the colloid substance. It was only after the 10th day that changes took place which would be noticeable in ordinary histological preparations fixed, for example, in Zenker's fluid and stained with hematoxylin and eosin. It is interesting to note that while the mitochondria retained their rounded form and occurred in about the same number from the 10th to the 25th day (Figs. 10 to 13), the nuclei commenced to exhibit unusual polymorphism which steadily increased up to the 45th day, as is shown in Figs. 12 to 15. During this later period, the mitochondria rapidly decreased in number

(Figs. 14 and 15) and continued to disappear to the 60th day, when the cells were distinctly swollen and the nuclear chromatin unusually granular (Fig. 16).

Approximately similar changes have been described in anemic necrosis of the kidney by Israel.[5] The negative results obtained by McCann[6] are perhaps to be explained by the peculiar and unusual resistance of mitochondria in nervous tissues to injurious influences and by the slow rate of autolysis as compared with glands like the thyroid and kidney.

Partial Extirpation.

For purposes of contrast and in order to investigate the effect upon the mitochondria of increased functional demand for thyroid secretion, the whole of one gland and half of that on the opposite side were removed in a series of five guinea pigs. They were sacrificed after 5, 10, 15, 20, and 25 days, respectively. It was found that while the mitochondria in the remaining fragment of tissue retained their usual form, they increased considerably in number. This may be seen by reference to Figs. 17 and 18.

These mitochondrial changes in experimental compensatory hypertrophy of the thyroid gland apparently differ from the alterations observed in exophthalmic goiter by Goetsch[4] in the failure of the individual mitochondrial filaments to increase in length, although in both conditions there is a marked increase in number. A corresponding increase in the number of granulations, probably of mitochondrial nature, has been observed in compensatory hypertrophy of the kidney by Enderlen,[7] Hirsch,[8] and De Giacomo.[9] It seems to be of rather common occurrence in regenerating tissues,[10] despite the fact that Oliver[11] has reported a decrease in the regenerating kidney cells of uranium nephritis.

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[5] Israel, O., *Virchows Arch. path. Anat.*, 1891, cxxiii, 310.

[6] McCann, G. F., *J. Exp. Med.*, 1918, xxvii, 31.

[7] Enderlen, E., *Deutsch. Z. Chir.*, 1895, xli, 208.

[8] Hirsch, C., *Anat. Hefte*, 1910, xli, 125.

[9] De Giacomo, A., *Internat. Monatschr. Anat. u. Physiol.*, 1911, xxviii, 208.

[10] Romeis, B., *Anat. Anz.*, 1913, xlv, 1.

Torraca, L., *Arch. Zellforsch.*, 1914, xii, 539; *Anat. Anz.*, 1914, xlv, 459; *Arch. ital. anat. e embriol.*, 1916–17, xv, 326.

[11] Oliver, J., *J. Exp. Med.*, 1916, xxiii, 301.

Effect of Thyroid Feeding, Fasting, and a Water-Soluble B Vitamine-Free Diet.

The mitochondria reacted quickly to the administration of whole desiccated thyroid gland substance.[12] After feeding it at frequent intervals during 1 day, they showed a tendency to round up into spherules (Fig. 19), which was more marked after 2 days. After 4 days, no filamentous forms remained and the total number had become greatly reduced (Fig. 20). The animals at the same time rapidly lost weight. It is evident, from a comparison of Figs. 20 and 12, that an end-result of the same general character as far only as the mitochondria are concerned, may be obtained by vessel ligation and thyroid feeding. But this does not mean that there is necessarily any fundamental parallelism in the mechanism of cellular response to these therapeutic measures, because, as we shall see, fragmentation and diminution of mitochondria are reactions exhibited under conditions of the most diverse kind. For example, after the daily subcutaneous injection of 1 cc. of 1 per cent potassium iodide solution in water, the mitochondria again lost their filamentous form and became spherical, though in this instance the animals gained in weight. The same result was obtained whether they were kept under the influence of potassium iodide for 10 days (Fig. 21) or 25 days (Fig. 22).

Quite the opposite was the effect of fasting. After 3 days the mitochondria lost their filamentous form (Fig. 23); after the 4th day there was a definite diminution in number coincident with the appearance in the cytoplasm of large round bodies which blackened with osmic acid (Fig. 24); while at the end of 6 days of fasting the mitochondria completely disappeared and the droplets blackening with osmic acid exhibited a further increase in number (Fig. 25). These results are in general accord with observations which have been made by several investigators[13] on the influence of inanition upon the mito-

[12] Prepared by Burroughs Wellcome Company, London, England.

[13] Russo, A., *Arch. Zellforsch.*, 1910, v, 173.
Azzi, A., *Arch. sc. med.*, 1916, xl, 22.
Ono, S. I., *China Med. J.*, 1920, xxxiv, Anat. suppl., 23.
Ma, W. C., *Anat. Rec.*, 1923, xxv, 157.
Okuneff, N., *Arch. mikr. Anat.*, 1923, xcvii, 187.

chondria in other tissues, although there is some difference of opinion as to whether the mitochondria completely disappear before death.

Eight rats fed upon a water-soluble B vitamine-free diet[14] showed in all cases a diminution in the number of the mitochondria of the thyroid gland and a rounding up into spherules, in confirmation of results previously obtained by Miller[15] and Wakefield[16] in the gastrointestinal epithelium and in the pancreas. Compare Fig. 27 with the normal for the rat as represented in Fig. 26. In this connection we may profitably bear in mind that the literature contains many references to rapid modifications in the shape and the number of mitochondria in simple aseptic autolysis, notwithstanding the fact that the nuclei may show no evident deviation from the normal.[17] An instance in point is shown in Fig. 28.

Effect of the Inhalation of Gases.

The mitochondria were also found to respond directly and with astonishing rapidity to the inhalation by the animal of various gases. Four guinea pigs were allowed to breathe almost pure oxygen in a glass chamber of about 2 liters capacity, with occasional return to air, for $1\frac{1}{2}$, 5, $6\frac{1}{2}$, and 9 hours, respectively. The mitochondria in each rounded up into spherules to about the same degree, irrespective of the duration of inhalation (Figs. 29 and 30).

In the administration of carbon dioxide, the animals had to be periodically revived by permitting them to breathe air at frequent intervals. Changes in the mitochondria were noted that were like those after oxygen as far as could be ascertained, and were also independent of whether the treatment was continued for $2\frac{1}{2}$, 4, 6, or $10\frac{1}{2}$ hours; but the alterations were not quite so pronounced, since a few rod-like mitochondria could still be found, as reference to Figs. 31 and 32 will show.

[14] Obtained through the kindness of Miss Muhlfeld.

[15] Miller, S. P., *Anat. Rec.*, 1922, xxiii, 205.

[16] Wakefield, H. W., *Anat. Rec.*, 1923, xxv, 158.

[17] Dannehl, P., *Virchows Arch. path. Anat.*, 1892, cxxviii, 485.
Takaki, K., *Arch. mikr. Anat.*, 1907, lxx, 245.
Biondi, G., *Virchows Arch., path. Anat.*, 1915, ccxx, 232.

A parallel, but perhaps less striking granular metamorphosis was discovered after the inhalation of hydrogen under the same conditions for interrupted periods amounting to $2\frac{1}{2}$, 5, and 7 hours (Figs. 33 and 34).

As a control measure, animals were allowed to breath just as little air as would suffice to maintain life for $2\frac{1}{2}$, 4, and $6\frac{1}{2}$ hours. Upon making preparations, the mitochondria were found to be unaltered, except that they evinced a tendency to be clumped about the nuclei (Figs. 35 and 36). Miller[15] also failed to detect mitochondrial changes resulting from asphyxiation. The alterations described by Champy[18] in tissue cultures, and attributed by him to asphyxiation, are difficult of interpretation.

Effect of Chemical Agents.

The following substances were deliberately selected as likely to modify cellular activity radically and in different ways: insulin, atropine sulfate, pilocarpine hydrochloride, adrenalin chloride, morphine sulfate, potassium arsenite, hydrogen cyanide, and phosphorus. All, except the insulin which was given to rabbits, were administered subcutaneously.

For experimentation with insulin, Dr. P. A. Levene very kindly placed at my disposal a series of eighteen rabbits which had received injections of about 20 mg. of insulin per kilo of body weight directly into the heart. They usually showed convulsions from 1 to 2 hours later lasting for 3 or 4 hours. Tissues were taken $\frac{1}{3}$, $1\frac{3}{4}$, 3, $3\frac{1}{4}$, $3\frac{1}{2}$, 4, $4\frac{1}{4}$, $4\frac{1}{2}$, $5\frac{1}{4}$, $5\frac{1}{2}$, $5\frac{3}{4}$, and 6 hours after injection. None of the specimens (Fig. 38) showed any noticeable departure from the normal (Fig. 37), which is interesting—à propos of theories to the effect that mitochondria are concerned in cellular respiration,[19]—in the light of the fact that insulin treatment causes a tremendous increase in the rate of oxidation. A similar result was obtained with three guinea pigs examined 6, 51, and 52 hours after repeated small subcutaneous injections of insulin. Compare Figs. 39 and 40.

[18] Champy, C., *Arch. zool. exp. et gén.*, 1914, liv, 307.

[19] Kingsbury, B. F., *Anat. Rec.*, 1912, vi, 39.

Mayer, A., Rathery, F., and Schaeffer, G., *J. physiol. path. gén.*, 1914, xvi, 607, and others.

Atropine sulfate was administered to two animals in doses of 0.001 gr. twice in 24 hours, followed by 0.02 gr. which killed them within an hour's time. Tissues were taken immediately and a change of some mitochondria into granular forms was noted (Fig. 41). Two other animals were given 0.01 gr. every day for 10 days which caused a reduction in body weight of about 10 per cent with a change in the mitochondria to the granular form (Fig. 42). Pilocarpine hydrochloride was given to two guinea pigs in 0.05 gr. doses, increased by 0.05 gr. each hour for 4½ hours, which likewise brought about a partial rounding up of the mitochondria (Fig. 43). Two others received 0.0625 gr. doses, increased by 0.0625 gr. each day, for 3 days and 5 hours, which proved fatal. In this instance the mitochondria were all changed to granular forms and their number greatly depleted (Fig. 44). Atropine and pilocarpine, then, substances which produce distinctly different physiologic responses in glandular tissues caused in the thyroid a similar rounding up of filamentous mitochondria into spherules.

1 cc. of 1:1,000 adrenalin chloride solution injected in the neighborhood of the thyroid gland killed an animal in 2 hours; and a close study of the tissues revealed an increase in size and a rounding up of mitochondria (Fig. 45). The experiment was twice repeated with similar results. When 1 cc. was injected subcutaneously, remote from the gland, in two guinea pigs daily for 3 days and 10 days, respectively, the same result was again obtained (Fig. 46).

Two animals were given 0.125 gr. of morphine sulfate daily for 10 days without noticeable effect upon the mitochondria (Fig. 47). Two others received 0.125 gr., increased by a like amount each day for 25 days, which caused a loss of 10 per cent in weight, some rounding up of mitochondria and a diminution in their number (Fig. 48).

Potassium arsenite, administered in 0.5 cc. of 1 per cent solution doses to two animals, brought about death in 5 hours with no detectable change in the mitochondria (Fig. 49). Two others received 1 cc. of a 0.1 per cent solution daily for 11 days which caused a loss of weight of 9 per cent and a barely noticeable alteration in the mitochondria in the direction of spherule formation (Fig. 50).

Hydrogen cyanide given in a dose amounting to 0.5 cc. of U.S.P. No. 9 to two animals resulted fatally in 15 minutes, without producing any

alteration in the mitochondria (Fig. 51). Another animal received 1 drop of HCN and a second drop at the end of 26 hours, and was sacrificed. Preparations of the thyroid again revealed no change in the mitochondria. Two others were given 1 drop, increased by 1 drop twice daily for 6 days, which killed them and produced a profound modification in the cell with almost complete disappearance of mitochondria (Fig. 52). Here again the time factor was evidently of importance.

Lastly, phosphorus was employed. A guinea pig given subcutaneously 0.5 cc. of a saturated solution in olive oil died in 21 hours. Preparations of the thyroid showed a granulation and swelling of the mitochondria and the appearance of the familiar droplets of fatty degeneration, which blackened with osmic acid (Fig. 53). Two others received doses of 1 cc. of the same solution, diluted with four parts of olive oil, daily for 5 days which killed them. There was a complete disappearance of mitochondria and a further increase in the masses that blackened with osmic acid (Fig. 54). With a view to further prolonging the time of treatment 0.5 cc. of the diluted solution was injected daily for 6 days which also resulted fatally, with loss of mitochondria and a tendency on the part of the blackened bodies to disappear also. It will be recalled that Scott[20] observed a similar fragmentation of mitochondria with the appearance of droplets of fat in the acinus cells of the pancreas of mice likewise poisoned with phosphorus. In addition, he discovered that the fat was formed through the clumping together, fusion, and metamorphosis of the mitochondria. The decrease in number of mitochondria had been noted several years earlier by Lubarsch,[21] who referred to them as Altmann's granules.

RESULTS.

The foregoing experiments have demonstrated the following constant qualitative and quantitative changes in the mitochondria in the thyroid glands of guinea pigs.

1. Fragmentation of Filaments into Granules.—This was the most common reaction encountered and was produced by many influences:

[20] Scott, W. J. M., *Am. J. Anat.*, 1916, xx, 237.

[21] Lubarsch, O., *Ergebn. allg. Path. u. path. Anat.*, 1897, iii, 631.

reduction of blood supply, feeding of thyroid substance, fasting and vitamine deficiency, the inhalations of oxygen, hydrogen, and carbon dioxide, and by the administration of atropine, pilocarpine, adrenalin, morphine, phosphorus, potassium arsenite, and hydrogen cyanide. It occurred with the greatest rapidity after breathing oxygen and not so readily after strong poisons like arsenic and cyanide. It was associated with both an increase and a decrease of body weight. The general similarity in response to influences of such divergent character is compatible with the hypothesis that the filamentous state of mitochondrial substance is dependent upon the maintenance of conditions which may be extremely easily disturbed in a variety of ways. It is very remarkable that no definite changes were observed as a result of the injection of insulin, which causes an acceleration of oxidative processes.

2. Reduction in the Number of Mitochondria.—This reaction was usually observed coincidently with the preceding, and in common with it, and may be often illusory resulting from faulty technique alone. The change was, however, noted with great constancy after reduction of the blood supply, the feeding of thyroid substance, on vitamine deficiency, and when pilocarpine, morphine, and hydrogen cyanide had been administered. It was also observed after phosphorus poisoning and fasting, but in these instances was concurrent with the appearance of fatty droplets within the cells. Such an association of mitochondrial loss with the appearance or non-appearance of visible fat indicates forcibly that a similar mitochondrial response may occur in opposed functional states.

3. Increase in the Number of Mitochondria.—Increase in the number was noted only in association with compensatory hypertrophy after removal of some three-quarters of the thyroid tissue. The shape of the mitochondria remained unaltered. In no case has it yet been possible to bring about experimentally an increase in the length of the mitochondrial filaments— that is to say, to produce a change such as Goetsch[4] discovered in exophthalmic goiters. In order so to do it would probably be necessary to experiment extensively along somewhat different lines.

EXPLANATION OF PLATES.

The drawings were made with the aid of a camera lucida, a Zeiss compensating ocular 8 and an apochromatic objective of 2 mm. They have been reduced one-third in reproduction so that they now present a magnification of 1,000 diameters.

PLATE 9.

FIG. 1. Specimen from an embryo shortly before birth, which showed mitochondria of normal morphology but slightly more abundant than in young adults. (*Cf.* Fig. 4).

FIG. 2. The many mitochondria observed 1½ hours after birth.

FIG. 3. 4 days after birth; mitochondria still more numerous than they are in young adults.

FIG. 4. Mitochondria in a young, healthy adult guinea pig of about 325 gm. taken as the normal.

FIG. 5. Tissue from an old guinea pig, showing mitochondria which were slightly shorter and less abundant than those of Fig. 4.

FIGS. 6 to 10. 3, 5, 7, 9, and 10 days after ligation of large blood vessels to gland, showed a progressive tendency on the part of the mitochondria to become shorter and more granular in form.

FIGS. 11 to 13. Specimens of the ligation series, procured after 15, 20, and 25 days, showed continuance of rounding up of mitochondrial filaments.

FIGS. 14 to 16. 35, 45, and 60 day stages, illustrative not only of a rounding up of the mitochondria into granules, but of the gradual lessening in their number.

PLATE 10.

FIGS. 17 and 18. The increase in number of mitochondria observed in the thyroid 10 and 25 days, respectively, after the removal of one gland and of half the gland on the opposite side.

FIGS. 19 and 20. The rounding up of filamentous mitochondria into spherules and decrease in number occurring after 1 and 4 days feeding of thyroid gland substance.

FIGS. 21 and 22. To show that the daily subcutaneous injection of potassium iodide in dilute solution for 10 and 25 days, respectively, brought about a loss of the normal filamentous shape and a decrease in size of mitochondria.

FIGS. 23 to 25. 3, 4, and 6 days of complete fasting caused a rounding up of mitochondria, a pronounced decrease in number, and the appearance in the cytoplasm of many large droplets which blackened with osmic acid.

FIGS. 26 and 27. Illustrating the normal appearance of mitochondria in the thyroid of a white rat and the decrease in their number which resulted from holding white rats upon a vitamine B-free diet.

FIG. 28. For comparison, the gland from an animal which had been dead for 7 hours, showing complete autolysis of mitochondria.

FIGS. 29 and 30. Effect of breathing pure oxygen alternating with air for 1½ and 9 hours. It caused a transformation of filamentous mitochondria into spherules.

FIGS. 31 and 32. Breathing carbon dioxide and air alternately for 2½ and 10½ hours produced comparable but less marked changes.

FIGS. 33 and 34. Breathing hydrogen under the same conditions for 2½ and 7 hours likewise caused a rounding up of mitochondria.

FIGS. 35 and 36. Animals allowed as control to breathe only sufficient air to keep them alive for 2½ and 6 hours exhibited no change in the shape or number of the mitochondria, but showed a tendency to clump about the nucleus.

PLATE 11.

FIGS. 37 and 38. To illustrate the appearance of mitochondria in the thyroid of a normal rabbit and the lack of response on their part to an injection of insulin directly into the heart 6 hours previously.

FIGS. 39 and 40. The mitochondria in the thyroids of guinea pigs were equally unresponsive showing no changes 6 hours after the subcutaneous injection of insulin or 52 hours after the repeated injection of small doses.

FIGS. 41 and 42. No alterations in the mitochondria were to be noted 1 day after the administration of a heavy dose of atropine, but the repeated injection of small doses over a period of 10 days caused all to become rounded into spherules with a slight lessening in their number.

FIGS. 43 and 44. After a dose of pilocarpine, the majority of the mitochondria became spherical in 4½ hours. Repeated doses over a period of 3 days and 5 hours caused all the mitochondria to become spherical while their number underwent a great reduction.

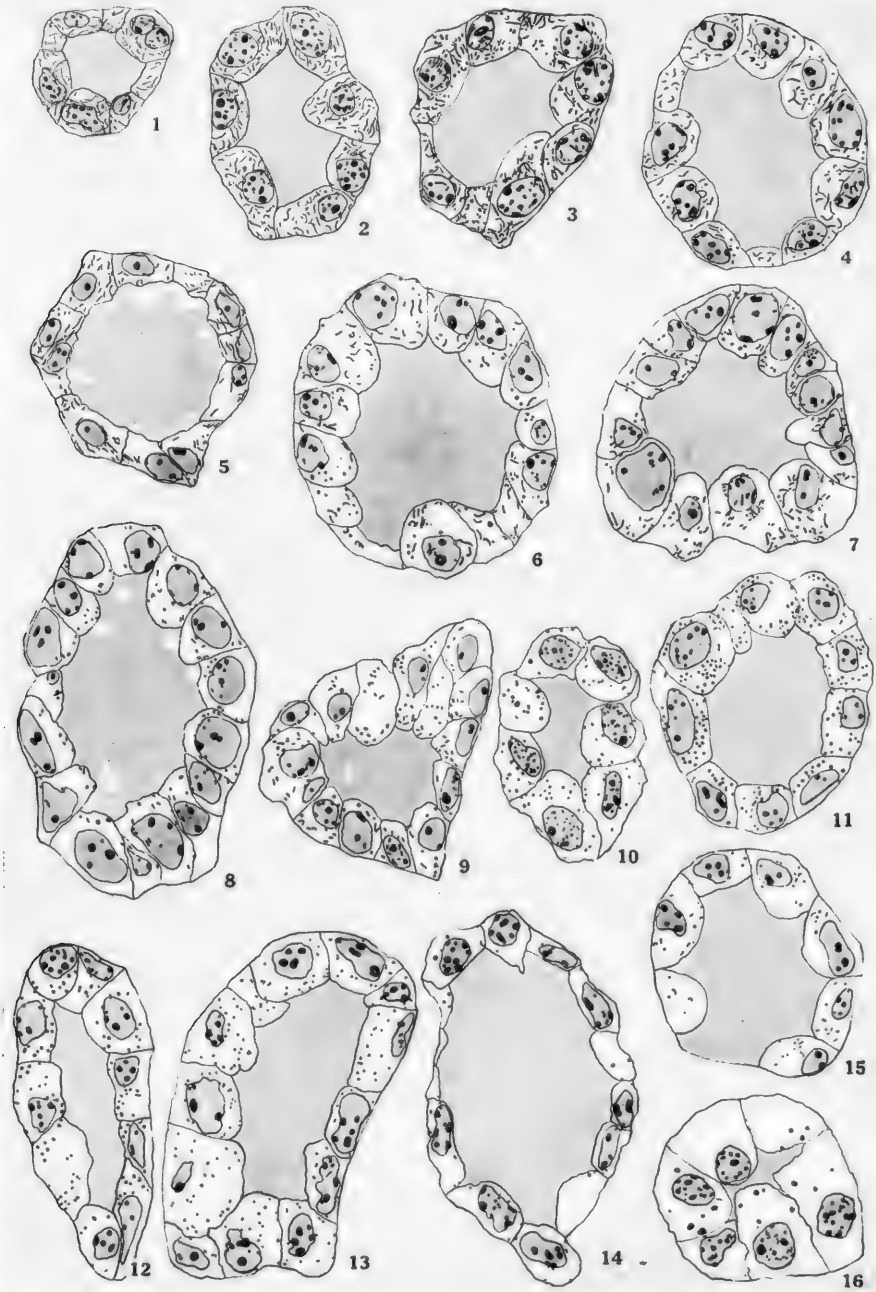
FIGS. 45 and 46. The mitochondria had rounded up and increased in size 2 hours after the injection of adrenalin as well as when smaller quantities had been injected daily for 10 days.

FIGS. 47 and 48. Daily dosage with morphine for 10 days produced no alterations in the mitochondria, but after the treatment had been continued for 25 days, they were distinctly more granular and slightly reduced in number.

FIGS. 49 and 50. With arsenic, no changes could be detected after 5 hours, but when smaller amounts had been given for 11 days, the mitochondria became spherical.

FIGS. 51 and 52. Similarly in the case of hydrogen cyanide, a lethal dose left the mitochondria normal whereas when the administration was distributed over 6 days a considerable depletion in number was brought about.

FIGS. 53 and 54. The injection, subcutaneously, of a solution of phosphorus in olive oil produced a rounding up and disappearance of mitochondria and the formation of numerous large droplets blackening with osmic acid.



(Nicholson: Mitochondrial changes in thyroid gland.)

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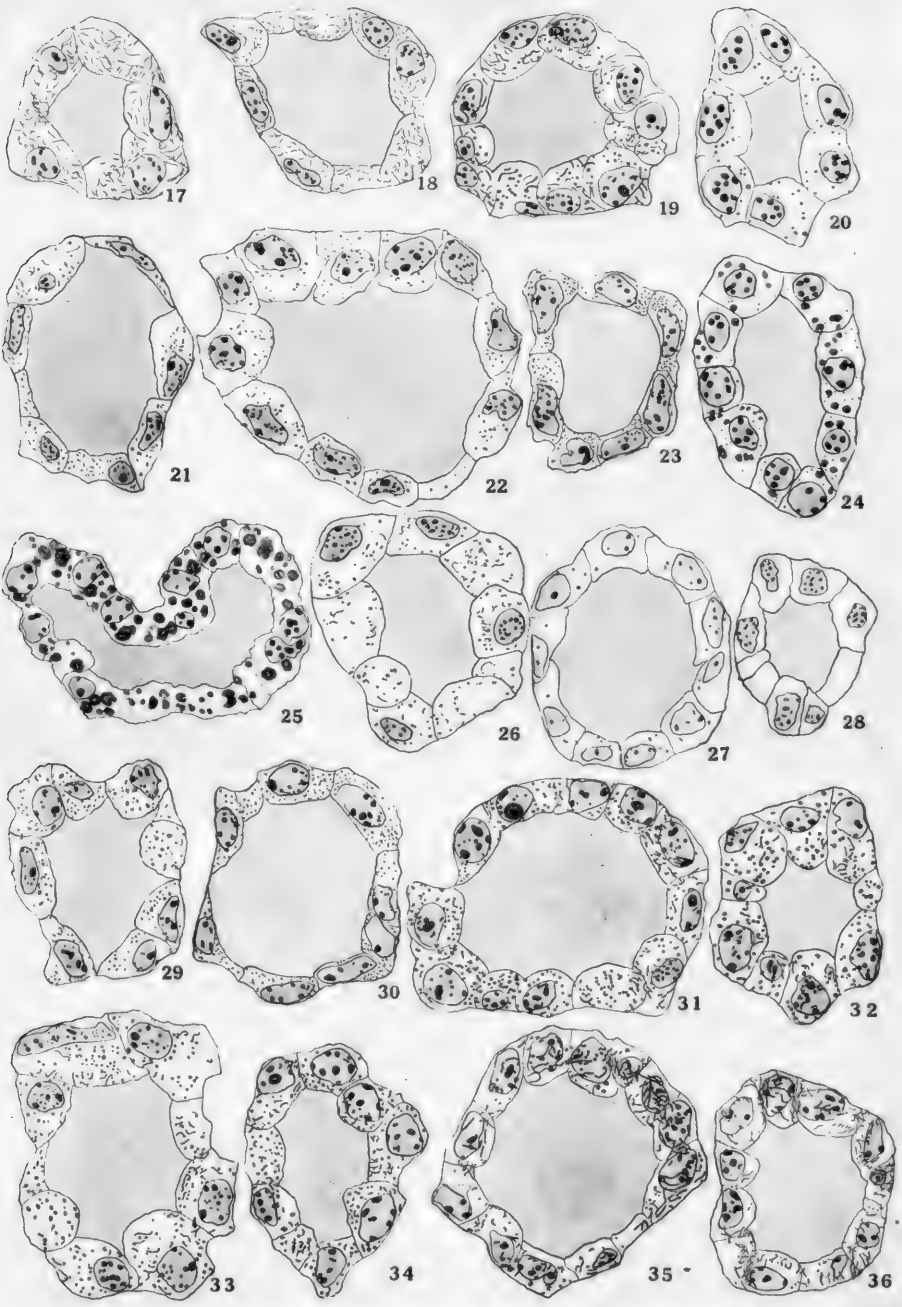

(Nicholson: Mitochondrial changes in thyroid gland.)

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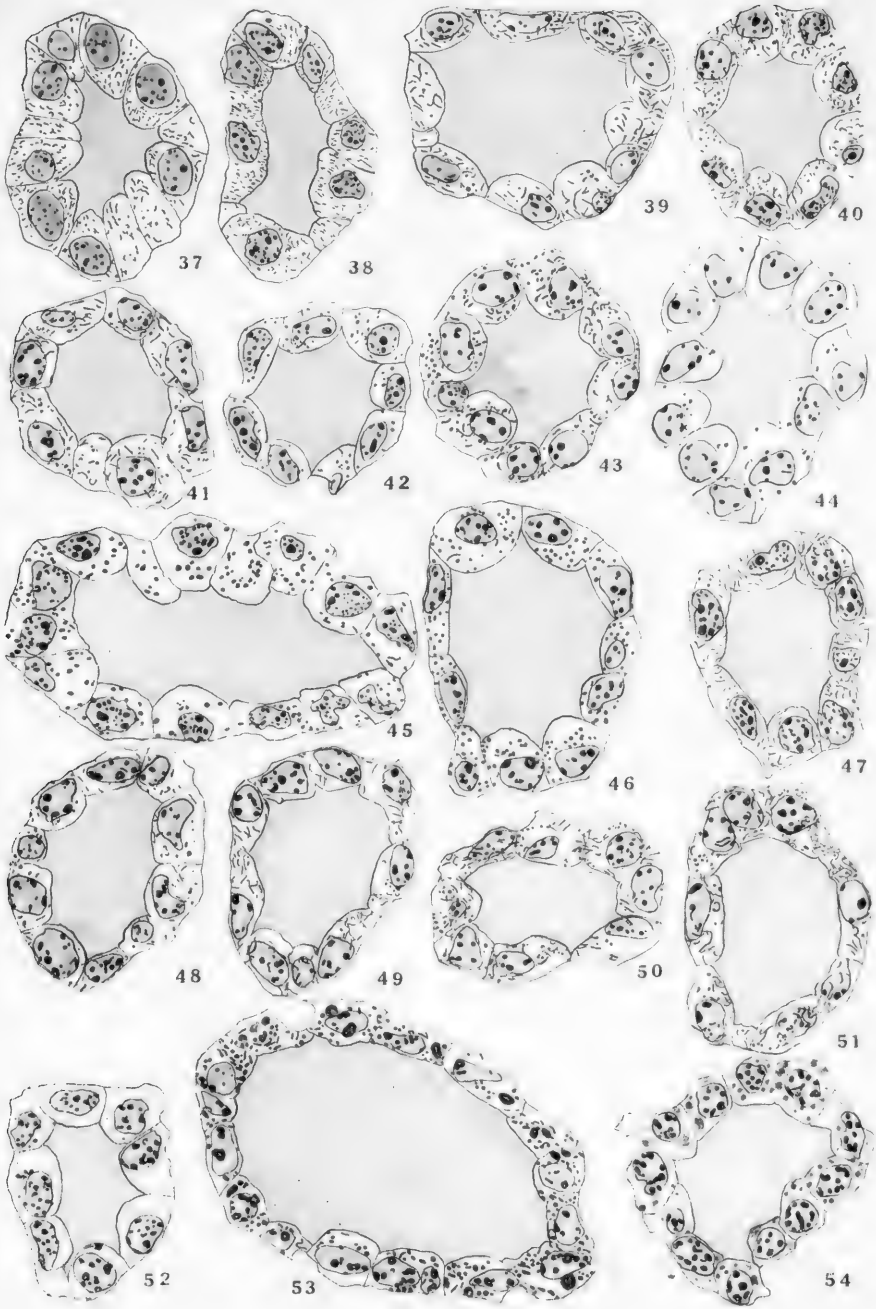

(Nicholson: Mitochondrial changes in thyroid gland.)

UNIV. OF MICH.

OBSERVATIONS ON SOME CAUSES OF GALL STONE FORMATION.

I. Experimental Cholelithiasis in the Absence of Stasis, Infection, and Gall Bladder Influences.

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Plates 12 to 15.

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In the present paper and others to succeed it we shall report experiments on the causation of gall stones. Whatever the significance of our findings, or lack thereof, there can be no doubt that the problem of cholelithiasis waits upon the development of an experimental material. The literature of the subject, like that of many another medical theme, exhibits the difficulties which sometimes effectually thwart attempts to solve a riddle in etiology by the study of clinical, which is to say uncontrolled, instances. It has been from early times a literature top-heavy with hypothesis and dismal with uncorrelated observations. The admirable studies of Naunyn, Aschoff and Bacmeister, and others which have so greatly furthered knowledge on the classification and incidence of stones have not explained their causation,—witness, for example, the prevailing uncertainty as to whether infection plays an essential rôle therein. Gall stones have frequently been encountered and produced in animals, but only under conditions of infection and biliary disturbance which render the circumstances of their occurrence not a whit more easy to understand than in man.

The lithiasis with which we have had to do developed in dogs permanently intubated for the collection of sterile bile.[1]

[1] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 11.

Most of the successful attempts to induce gall stones in animals have been carried out with the herbivora (rabbits, guinea pigs). This is not for lack of work with species such as the dog which approach man more nearly in their physiological processes; but in them calculi have proved difficult to engender, for reasons as yet uncomprehended. Stadelmann[2] mentions stone formation upon the surface of a broken cannula left in a dog with a bile fistula. Gilbert,[3] Gilbert and Fournier,[4] and Mignot[5] all caused cholelithiasis in dogs by infecting the gall bladder with organisms of low virulence and hindering the evacuation of bile. They were interested only in the relationship of infection to the lithiasis and give no detailed description of the stones,—nor for a like reason does Rosenow[6] who has reported stones as a consequence of the elective localization of streptococci in the canine gall bladder. Harley and Barratt,[7] and Hansemann,[8] following Naunyn, introduced cholesterol stones from man into dogs and saw that instead of increasing in size they gradually dissolved away, a fact explained by the low content in cholesterol of dog bile.

Gall stones developed in fourteen out of twenty-two intubated dogs which we have studied with reference to the phenomenon. Of the eight animals free from them, one had been intubated for only 7 days.

The make-up of the tube system has already been described.[1] It consisted of a glass cannula (Fig. 1, *A*) 1 to 2 cm. long with a bore of 1½ to 3 mm., joined to a U of rubber tubing measuring 7 to 10 cm. on a side. The sides of the U were connected by a short, curved segment of glass (Fig. 1, *B*), and that nearer the duct consisted of soft, black rubber while the further one was of more resistant red material. All this portion of the apparatus lay within the peritoneal cavity, but the red tubing was prolonged to pass out through a stab wound in the abdominal wall and connect with a rubber balloon into which it delivered the bile. Close to the balloon, as a provision to empty it, a side tube was incorporated in the system by means of a T of glass (Fig. 1, *C*). The total length from cannula mouth to balloon ranged between 25 and 30 cm. Small bulbs had been blown in the glass connections used in some cases, to facilitate deposition out of the bile. Prior to assembling the apparatus, the rubber segments were boiled in a sodium bicarbonate solution, soaked in weak hydrochloric acid, and repeatedly

[2] Stadelmann, E., Der Icterus und seiner verschiedenen Formen, Stuttgart, 1891.

[3] Gilbert, A., *Arch. gén. méd.*, 1898, x, series 8, 257.

[4] Gilbert, A., and Fournier, L., *Compt. rend. Soc. biol.*, 1897, iv, 936.

[5] Mignot, R., *Arch. gén. méd.*, 1898, x, series 8, 129.

[6] Rosenow, E. C., *J. Infect. Dis.*, 1916, xix, 527.

[7] Harley, V., and Barratt, W., *J. Physiol.*, 1903, xxix, 341.

[8] von Hansemann, *Virchows Arch. path. Anat.*, 1913, ccxii, 139.

flushed with distilled water. The glass connections were cleaned with aqua regia and well rinsed. And finally the apparatus was autoclaved twice in a large flask of distilled water.

Incidence of the Stones.

The bile out of which the stones formed was liver bile. For the neck of the gall bladder has been severed between ligatures at the initial operation or tied off at several points, effectively isolating it from the duct system, as proven at autopsy. Stasis also can be ruled out as a factor in the lithiasis. It is true that temporary obstruction to the flow from the liver was designedly produced in certain of the animals[9] by clamping the collecting tube, and that on other occasions brief obstruction occurred through accidental twists in the tube; but an analysis of the incidence of calculi has failed to bring out any influence thereon of the stasis, which lasted at most only 48 hours. In many of the dogs developing stones, the flow of bile was never interrupted.

Thirteen of the animals were kept with special reference to maintenance of health, procedures likely to disorder them being avoided. Stones developed in seven of these, and in four of the number the bile was consistently sterile on day-to-day culture, while staining of the sediment thrown down with the centrifuge failed to disclose organisms. The secretion had become infected in four of the six animals which did not show stones at autopsy. Nine intubated dogs were submitted to procedures that may have, and often undoubtedly did, produce pathological changes in the secretion, as for example, the intravenous injection of hemoglobin or calcium chloride, prolonged chloroform anesthesia, and toluylenediamine poisoning. In all save two such animals, one intubated for but a few days as already mentioned, stones occurred. The bile was infected in two of the seven instances of lithiasis and in one of the two dogs that were free from stones.

The diet of the animals was so varied that the question of its influence must remain an open one.

The organisms recovered from the infected animals were always found in pure culture. They differed greatly in character. Some

[9] McMaster, P. D., Broun, G. O., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 395.

strains may well have acted to prevent or determine the occurrence of calculi, though they produced no obvious disorder of the biliary tract. In the lack of precise information on the point, attention will be concentrated on the lithiasis developing under sterile conditions.

Stones were found as early as 14 days after intubation. The longer the period of intubation, the larger in general they tended to be. In three cases in which the bile was sterile they led to total biliary obstruction after 21, 24, and 50 days intubation, respectively, through gradual closure of the lumen of the cannulas wherein they were situate.

Location.

Always the calculi were restricted to the system of glass and rubber tubes that had been connected with the common duct (Figs. 1 and 2). They were never found in the biliary system proper. This was true even when they gave rise to obstruction and the ducts were in consequence dilated, and also when, as not infrequently happened, there was a circumscribed irritation of the duct mucosa where the lip of the cannula had rubbed upon it. Even the cul-de-sac resulting from ligation of the gall bladder neck was regularly free from stones. They were sessile upon the foreign tubing; multiple; discrete in inception though often secondarily merged; and larger and more numerous the nearer their position to the bile source.

When the calculi were small they were sometimes present only in the cannula. Usually, though, other and smaller ones were to be found on the adjoining rubber tube. When those in the cannula were large, the incrustation was usually to be noted in diminishing amount for some centimeters further down, occasionally as far as the glass T-tube (Fig. 1), that is to say more than 20 cm. from the common duct. The alternation of glass and rubber surfaces was without obvious influence on the number or character of the stones; but wherever there was an eddy in the bile current or a shallow dead space, as just below the constriction in the cannula and at the junctions of rubber and glass, they were relatively numerous and large (Fig. 2). They were never found, significantly enough, on the outside of that portion of the cannula which was included within the duct by the ligature, nor, save once, were any present at the immediate entrance to the cannula or on the first few millimeters of its wall, in contrast to the state of affairs existing further down (Fig. 1). In the instance referred to, an occluding calculus in the cannula projected upwards as a jagged mass into the duct. Inspection showed that it had had its inception at a point several millimeters below the cannula mouth.

The stones were always relatively large and abundant on the side of the tubing that was lowest during the life of the dog (Figs. 1 and 2), and frequently were found only there. The materials of which they were principally composed caused them to be much heavier than the bile. Those near any one point tended to be remarkably uniform in size and constitution, as if of the same "crop" or "generation" (Figs. 3 to 5). In exceptional instances a second and even a third "crop" could be discriminated, scattered amidst the first (Fig. 1). The deposition was punctate to begin with, the stones enlarging as separate entities until they touched each other when they often became secondarily united in a matrix. Ultimately the cannula became lined in some cases with a rigid tube of calculous matter, the merged entities of which could still be recognized here and there.

A considerable length of the tube system lay outside of the body, and the bile coursing through it must have been cooled somewhat. An influence of the factor on stone formation and localization could not be discerned, but that is scarcely surprising since deposition out of the bile had spent itself close to the duct. Furthermore the outside tubing was handled so often as almost of necessity to dislodge incrustations upon it.

Structure.

The calculi were found to be unaffected by water, so the tube system was gently rinsed out prior to scrutiny of them. The early stages in their development and association could be studied by direct inspection with the microscope through the walls of the glass connections. Those on the rubber were scraped off into water for examination. In general the stones exhibited little variety of structure and composition as compared with human stones. Those situate nearest the bile source were regularly the most pigmented, being usually of an opaque brownish or greenish black to the unaided eye, like small, angular pepper grains (Fig. 1). In an unique instance a few calculi having place on the side of the cannula which was uppermost during life resembled small, dark brown stalactites with prolonged points, in contrast to others, on the lower side, which had the usual angular contours and were but lightly pigmented (Fig. 6). Not infrequently calculi situate far down in the tube system were almost colorless, and in one instance this was true of those in the cannula itself. They appeared mustard yellow, like *Lycopodium* seeds in the gross.

The slightly pigmented stones showed evidence of crystalline structure (Figs. 6 and 7) and even when relatively large sometimes retained the crystal shape, appearing like flattened rhombs or plates (Figs. 8 and 9). Arrow-head forms and forms like angular pears were frequent. The admixture of pigment evidently interfered with the crystallization; and many of the heavily colored stones, as for example the stalactites above mentioned, appeared to consist of a nearly amorphous material. Concentric layers of varying degrees of pigmentation could frequently be made out. The center of many of the stones was more pigmented than the periphery, often pronouncedly so (Figs. 6 and 10). On the other hand a deposition of colored layers on a non-colored core was never encountered. Whatever the composition of the stones, they were nearly always hard and brittle, grating under the knife, and shattering into many fine splinters when forcibly pressed. Only occasionally were small concentric yellow-brown forms noted (Fig. 11) that appeared less rigid, separating on pressure along radial and concentric lines into pieces with rounded corners.

A specimen example of the cholelithiasis will be described:

Dog 30, a male terrier of 18½ kilos, was killed with chloroform on the 38th day of bile collection. The secretion had remained sterile throughout. At autopsy the ducts were somewhat more distended than is normal, though the walls were not stretched. On an attempt to run saline through the tube system, from above downwards, a slight resistance was met which was relieved by the expulsion of a plug of soft mucus.

Stones.—The upper portion of the cannula was clean, save for a thin, transparent layer of mucus; but the lower part was thinly coated with organic débris and in its midst was a tongue-shaped group of calculi that averaged about ¼ mm. in diameter. They were roughly spherical, with an almost black, well defined, rounded nucleus and a well-nigh colorless, narrow, surface layer. Those nearest the duct were separate, but others a little further down on the glass were secondarily united in a pale green, translucent crystalline matrix. The wall of the rubber tubing next the cannula was encrusted all around with a similar deposit, lying in a layer of organic matter; but lower, and in the curved glass connection between the two limbs of the U, stone formation was limited to the dependent side of the tube, as also was the organic débris. The stones had the form of pale green, translucent, crystalline masses, some with relatively small, dark centers. When crushed, they separated into numerous jagged splinters and gave no evidence of concentric or radial structure. On the dependent side of the red rubber near where it was attached to the glass connection a bright brown skim was to be seen

which when scraped into water was found to consist of minute, spherical or ovoid, red-brown bodies caught in organic débris. The smallest were of about the size of an erythrocyte. The larger showed concentric layers of deposition and radiating striæ (Fig. 11). They broke readily on pressure along both the concentric and radial lines, and the fracture angles appeared somewhat rounded as if the calculi were soft. Further down were no stones but only a little organic débris. The plug of mucus flushed from the tube at autopsy showed a few calculi that had evidently been caught and dislodged in its passage.

Composition.

Calculi from different levels in the tube system of the first six animals with lithiasis were scraped into water, washed by centrifugation, and tested for bilirubin, phosphates, cholesterol, carbonates, calcium, and calcium bilirubinate. Less extensive analyses were carried out with the later material.

The method of analysis follows:

The washed material was dried on a watch-glass, powdered in an agate mortar, twice extracted in an excess of boiling water, and separated therefrom by sedimentation. Often some floccular débris failed to come down, and this was collected on a filter and tested for cholesterol (Liebermann-Burchard reaction). The sediment after drying was twice extracted with an excess of boiling chloroform (Merck's reagent), which was in turn filtered off and examined for the presence of bile pigment and cholesterol. After the extracted material had again been dried, separate portions were tested for calcium (oxalate reaction), for phosphates (with ammonium molybdate), for carbonates (barium hydroxide), and for calcium bilirubinate. The reactions of this last substance find scant mention in the literature on gall stones. According to the original description of Städeler,[10] it differs from bilirubin among other respects in being insoluble in chloroform, but is readily broken up by the action of weak acids, the bilirubin liberated going into solution when chloroform is present.

Carbonates were present in some of the stones from every animal as shown by the fact that they effervesced with weak hydrochloric acid and that the gas evolved caused clouding of barium hydroxide. None of those examined for phosphates contained them, and cholesterol was also absent, save in one instance in which the trace found was evidently derived from pus about the exit wound some of which had been inadvertently drawn into the tube system at autopsy. A trace of green pigment came away not infrequently in the watery extract of the stones, but the bulk of the pigment of those that were dark, and amorphous failed to go into solution in either water or chloroform. After treatment of them with weak acid, which evoked slight or no effervescence, almost all of the coloring

[10] Städeler, G., *Ann. Chem.*, 1864, cxxxii, 323.

matter came away into chloroform, conferring on the solvent an intense yellow-brown hue which changed gradually to green on exposure to the air. The pigment gave the Gmelin reaction brilliantly. In the residue after the extraction much calcium was regularly demonstrable. The inference seems justified that the original pigment complex was calcium bilirubinate. There was often present, in addition, some brown coloring matter which could not be identified (bilihumin?). There exists, of course, the possibility, indeed the probability, that the stones contained a little uncombined bilirubin which had come down incidentally in the precipitation of calcium carbonate, and was protected from solution in chloroform by this latter. The amount, though, was certainly negligible as compared with that of other pigment in the dark calculi.

Types of Stones.

Three general types of stones could be discriminated, one consisting almost wholly of calcium bilirubinate, another of calcium carbonate, and a mixed type in which both compounds were present. The composition and form of the stones varied, ordinarily, with the distance from the bile source. The relatively large, opaque, and deeply pigmented amorphous stones sometimes found in the cannula and upper tubing contained much bilirubinate; those lower down in the same animal showed usually a nucleus or core in which the substance predominated, enclosed in a more or less translucent matrix of concentric layers of tinted carbonate which gave some indications of the crystal form; while the lowest ones in the collecting system were frequently of almost pure carbonate, markedly crystalline in character, and scarcely pigmented at all. In one instance already mentioned, carbonate stones were alone found throughout the tubing, irregular crystalline masses, mustard yellow in the gross, of greenish tint under the microscope, and yielding on analysis a negligible amount of pigment. In another the minute calculi present side by side in the cannula were of two markedly different sorts, some of them being heavily pigmented, dark and amorphous, while others were translucent, nearly colorless, and obviously crystalline (Fig. 7). These latter dissolved in weak hydrochloric acid with a marked effervesence of carbon dioxide, and the solution yielded much calcium; whereas the pigmented elements scarcely effervesced at all, altered little in appearance, but were softened as shown by the fact that they now spread out on pressure instead of splintering. The stalactites situate on the upper side of the cannula in an instance previously referred to

consisted almost wholly of bilirubinate (Fig. 6, *c*), whereas the roughly crystalline elements on its lower surface contained, some of them, well-nigh pure carbonate, others a pigmented center and a carbonate envelope (Fig. 6, *a*, *b*, and *d*). No stones of a structure definitely radial were ever observed though scattered radial striæ were sometimes to be seen (Fig. 11). The varied forms of the calculi can be explained on the basis of the relative amounts of their two main constituents, the amorphous, calcium bilirubinate, and the crystalline carbonate. The organic débris in which some of them originated may sometimes have played a part in determining their shape (Fig. 6, *d*). Every stone, no matter how little colored, and how crystalline in appearance, possessed an organic scaffolding or shadow as could be seen by dissolving out its major constituents, which was readily accomplished with acid alcohol (Fig. 5). This shadow possessed the exact form of the original stone. On pressure it flattened out like mucus, which it resembled in appearance, returning to approximately its original shape when the tension was released. Not infrequently a glassy skim of mucus could be seen in a half light overlying the surface of calculi (Fig. 6, *c*, *d*),—the outermost, freshest layer of the scaffolding.

The Stones Not a Result of Bile Deprivation.

Total loss of the bile over long periods of time, such as occurred in our animals, may well be productive of grave changes in the general metabolism. Certainly there are radical alterations in the bile itself, practically from the first. The amount of it is lessened owing to absence of the normal stimulus to secretion from cholates delivered into the intestine and reabsorbed therefrom; and the total pigment content is cut down through the prevention of a like enterohepatic circulation of it.[11] If the stones we have described are the result of these and other biliary abnormalities, then they possess merely a curious interest. To determine the fact, recourse was had to experiment. Short segments of the common and hepatic ducts of dogs were excised and replaced with sections of glass tubing in which small bulbs had been blown. This was done with as little trauma to the duct system as possible and with no disturbance of its intes-

[11] Broun, G. O., McMaster, P. D., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 699.

tinal connection. The gall bladder was not interfered with. The operations were carried out by an aseptic technique, under ether.

Seven animals were thus treated. The abdominal wound healed by first intention in all. One dog died of an acute pneumonia after 27 days. At autopsy a few hours later, stones of the familiar, pepper grain type and consisting of mixed carbonate and bilirubinate were found in the single glass connection that had been inserted into the duct. Unfortunately no cultures of the bile were taken. There was no obstruction or inflammation of the duct system and no definite liver change. The glass connection had been placed in the large main duct from the liver just above the point where by entrance of the tributary from the lateral hepatic mass (made up of the right lateral and caudate lobes) it becomes the common duct.

In two dogs biliary obstruction with jaundice developed after some weeks as the result of complications resulting from the presence of the glass tubes. One animal, killed 29 days after operation, and presumably after a week or more of obstruction, showed no calculi. Tubes had been inserted into the common duct and that from the left lateral and central liver lobes. The obstruction had occurred in the tube first mentioned, through clotting within it of blood from the abraded duct wall. The tarry bile which filled to distension all of the ducts and the gall bladder proved sterile on culture. The other animal was killed when heavily jaundiced, 30 days after operation. It had by contrast a heavily infected bile and a liver which showed scattered small necroses. The lower end of a glass tube originally placed in the common duct was found to have worked through the wall and now lay in an occluding blind sac of scar tissue. Both in this tube and in another lying in the course of the duct from the lateral mass of the liver there were present lightly pigmented carbonate concretions of characteristic crystalline appearance, scattered here and there in the midst of a heavy coat of cellular débris.

Three animals, sacrificed after 29, 31, and 66 days, respectively, yielded no calculi. The one kept for the longest period had a bile heavily clouded with a pure culture of micrococci. The gall bladders of the others yielded a normal-looking secretion that proved to be sterile. In a final instance, typical carbonate stones were found associated with organic débris in a tube placed in the common duct of an animal killed while in excellent condition 35 days after operation (Figs. 8 and 9). The bile proved sterile on agar and bouillon culture, as, furthermore, on microscopic examination of the slight sediment obtained by centrifugation.

These experiments suffice to prove that the stones developing in our intubated animals were not essentially consequent on the alterations in the bile involved by loss of this secretion from the body.

Relation of Organic Débris to Stone Formation.

In twelve out of the fourteen intubated dogs in which lithiasis was encountered organic débris was found closely associated with the stones. Only twice did they develop when it was absent, which was the case in eight animals. The débris took the form of a thin coating on the tube surface of translucent or transparent, slightly granular and pigmented material containing occasionally a few degenerated cells. In some instances an obvious source for the material was discovered in a local irritation of the common duct where the lip of the cannula had rubbed. Sometimes the mucosa was raised here into a reddened papilla. In other cases in which the liver had been damaged with chloroform or toluylenediamine, the centrifuged bile of the day-to-day specimens showed desquamated cells in addition to much mucus, and the organic coating on the tubes at autopsy was thicker than usual, often extending far below the region in which the stones were located.

The nature of the association between the organic débris and the calculi became clear on a microscopic inspection of the latter *in situ*, on the walls of the cannula and the glass connections. In an instance in which small calculi of two sorts existed in the cannula, carbonate concretions and others predominantly of bilirubinate, it was noted that only those of the carbonate had an association with débris. At the center of small individual masses of this latter lay one or several stones (Fig. 7). One might suppose either that the calculi had been secondarily enclosed in the organic material or that it had to do with their inception. But if they were enclosed, why had not this happened to the bilirubinate calculi intermixed with them? In another case, already several times mentioned, there existed on the dependent side of a cannula an organic layer deep within which layered carbonate stones were present. On the upper side of the same cannula were relatively large stalactites of bilirubinate, by contrast practically free from organic débris (Fig. 6). A search through all the instances of lithiasis has disclosed the fact that calculi formed primarily of carbonate were only once observed in the absence of the débris, whereas those of bilirubinate were often

completely free from it, and in other instances only seated or lodged upon it (Fig. 7) as if their original nuclei of deposition had been caught and retained by it. In one unusual case, already mentioned, all of the calculi in a tube system consisted of carbonate. There was present in this instance an unusual amount of organic débris. Altogether the facts indicate that the slight amount of organic material settling in the tube system played an important rôle in determining the formation of carbonate calculi. These never developed on the surface of the material but always within it as if determined by some special condition there. Occasionally carbonate crystals were found scattered diffusely through the layer of organic débris. Frequently this latter extended far below the zone of stone formation, —a fact which sufficiently proves that the calcium salts were derived not from it but from the bile.

The Rôle of Nuclei in the Stone Formation.

By treatment with acid alcohol the stones could be rid both of pigment and of calcium salts (Fig. 5) and their scaffolding subjected to direct inspection for special centers of deposition. In a number of instances staining was done to disclose bacteria but none were found when the bile itself had been sterile; and when it had become secondarily infected,—for it was never so in the first days of bile collection, —the central portions of the scaffolding proved free from the organisms that were abundant in the peripheral regions. The association of the carbonate stones with organic material has already been stressed. In but a single instance were calculi of this composition, devoid of special centers of deposition, found upon clean tubing. Occasional stones were observed which had particles of talc from the rubber at their center. But most interesting were the many which contained minute, rounded, pigmented bodies, so different in appearance from the general substance of the calculus as to suggest that they had a special origin, and so located within it that they might well have acted as centers of deposition. These were found in stones of all sorts but especially in such as contained much bilirubinate. Their presence could be brought out by treatment of the calculus with acid alcohol, for although much of their substance dissolved, they left

discrete, heavily pigmented shadows deep within the general scaffolding of the stone. In one case, that of Dog 30, already given in detail, we were fortunate in finding elements identical in appearance with these "nuclei" free on the wall of the lower portion of the tube system as well as within stones there and higher up.

It may be recalled that in this animal, Dog 30, minute red-brown bodies (Fig. 11) were present on the surface of the rubber below the glass connection in the U-tube. The smallest of these were the "nuclei" referred to. They were rounded, rather soft, and highly refractile. On treatment with a dilute watery solution of hydrochloric acid, they effervesced mildly and lost in refractivity but otherwise did not alter greatly in appearance, showing that they contained but little carbonate. About many of them a concentric deposition of mixed bilirubinate and carbonate had taken place (Fig. 11). When subjected to acid alcohol, they left discrete dark shadows. Similar shadows were recognizable after the alcohol treatment, deep within certain of the large stones from close to the bile source, stones which had bilirubinate cores and carbonate peripheries of varying thickness. Other of the stones, translucent, pale green, crystalline masses of almost pure carbonate, found in the usual association with cellular débris, did not contain them.

In the instance described, some of the stones were so small that the nuclei could be directly perceived within concentric layers of deposit. That they had served as centers of the deposition was evident. The most that could be said of those within the larger calculi of this and those in other animals was that they had place deep within the more or less homogeneous scaffolding left after the treatment with acid alcohol. Such treatment tends to destroy even well marked lines of concentric deposition, as we have noted repeatedly in the case of bilirubinate calculi overlain with carbonate.

The character and rôle of the nuclei will be more closely scrutinized in a succeeding paper. We wish to emphasize here merely the fact of their existence and of their evident relationship to the development of some stones.

DISCUSSION.

The findings make plain the fact that gall stones can on occasion be formed in the absence of influences to which many authors have accorded a primary significance in their etiology. We refer to infection, stasis, and gall bladder activity.

Gilbert,[3] Mignot,[5] Miyake,[12] Cushing,[13] and others, working in the nineties of the last century, were able to induce cholelithiasis in animals by combining injury to the gall bladder with infection. Their demonstration in rabbits, guinea pigs, and dogs was so conclusive as perhaps to have overshot the mark. It has led to a general belief that without infection stones fail to occur. But recently Kehr[14] has observed stone formation upon the free ends of threads left at operation within the human gall bladder under conditions of surgical asepsis; and Kretz has recorded a like happening in rabbits and dogs.[15] Aschoff and Bacmeister[16] report the occurrence of stones of special type in uninflamed human gall bladders which they suppose never to have been infected. These authors maintain that one type of human lithiasis is due to disturbances in the general metabolism, a view that has been actively combated, notably by Lichtwitz[17] and Naunyn.[18]

No one attempts to deny that infection of the biliary system is a frequent cause for gall stones; and intermittent biliary stasis is conceded by all to have an important influence thereon. Whether stasis acts merely by facilitating infection or by favoring deposition out of the bile has been a moot point. The remarkable concentrating activity of the gall bladder,[19] which finds special scope when the bile is retained long, may be recalled in this connection. Naunyn showed that concentrating human bile as such does not result in increased deposition from it; but on the other hand, he furnishes instances of the rapid growth of stones lodging in the common duct which tend to encourage a belief that the concentrating ability of the gall bladder plays, on occasion, a major rôle in lithiasis. He and others have emphasized the importance for stone formation of a pathologic bladder mucosa. Yet the frequency of intrahepatic stones in the human liver[20] shows that the rôle of the organ is no indispensible one.

Riedel has remarked that wherever bile passes, gall stones may form; and he has produced striking illustrations in point.[21] Yet it is none the less surprising to find, as we have in the present experiments, that there exists a marked tendency to precipitation out of

[12] Miyake, H., *Mitt. Grenzgeb. Med. u. Chir.*, 1900, vi, 479.

[13] Cushing, H., *Bull. Johns Hopkins Hosp.*, 1899, x, 166.

[14] Kehr, cited by Hewlett, A. W., Pathological physiology of internal diseases, New York, 1916.

[15] Kretz, R., in Krehl, L., and Marchand, F., Handbuch der allgemeinen Pathologie, Leipsic, 1913, ii, pt. 2, 507.

[16] Aschoff, L., and Bacmeister, A., Die Cholelithiasis, Jena, 1909.

[17] Lichtwitz, L., *Ergebn. inn. Med.*, 1914, xiii, 1.

[18] Naunyn, B., *Mitt. Grenzgeb. Med. u. Chir.*, 1921, xxxiii, 1.

[19] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 47.

[20] Beer, E., *Med. News*, 1904, lxxxv, 202.

[21] Riedel, *Berl. klin. Woch.*, 1901, xxxviii, 1.

normal liver bile. The point immediately comes up, why did not stones form within the bile passages of the intubated animals? The ducts possess a musculature and are far from being fixedly distended like our tubing. They possess the ability to rid themselves of organic débris, such as, collecting on glass and rubber, favors a formation of calculi. Furthermore, they elaborate a secretion of their own,[22] which, if it does nothing else, must act to keep their lining cleansed. That it may perhaps exert a direct influence to prevent lithiasis is suggested by the absence of stones from the immediate mouths of the cannulas of our animals (Fig. 1), in exception to the general rule that stone formation on the tubing was especially great at situations close to the bile source.

The solubility of calcium carbonate is affected by slight changes in the reaction of the fluid in which it is contained. *A priori*, one might suppose the deposition on our tubing to have been determined by a diffusion of alkali from the glass or rubber. The precautions taken to remove soluble matter from the tubing have been described, yet some may still have come away gradually into the bile. Perhaps the best evidence against such a cause for the lithiasis lies in the failure of carbonate calculi to form when the tube surface was free from organic débris. A further indication that the character of the tubing was of little consequence is to be found in the fact that the calculi from adjoining surfaces of glass and rubber were alike in character and size.

Stones were absent not only from the ducts of all of the animals, but from the tube system as well, of some that had been long intubated. This was true of two instances yielding sterile bile for 49 and 85 days, respectively, and of a third, intubated for 86 days, the secretion of which had become infected after a time. The shortest of the periods mentioned was more than sufficient for the development of good sized stones in other animals, and sometimes even of occluding ones. It is true that the tubing was free from organic débris in the three exceptional cases, but so, too, was that of a dog already referred to in which discrete carbonate stones, lacking evident centers of deposi-

[22] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 75.

tion, were found after 46 days of bile collection. The conclusion seems justified that individual differences in the character of the bile must sometimes have determined the occurrence of calculi.

Human gall stones are of far more varied character than those here described for the dog, as would follow from the diverse pathological states in which they have been noted to develop. It seems wholly likely from the established relation between peculiar local conditions and human stones of special character [16,18] that, if said conditions were as narrowly controlled in man as in the experimental animals of the present work, the resulting lithiasis would be quite as uniform in type.

Gilbert[3,4] and Mignot[5] seem to have assumed that the stones developing in their dogs under the joint influence of local infection and tissue injury consisted of cholesterol; but they present no data on the point. Dog bile contains relatively little cholesterol.[23] The substance never, so far as we have been able to discover, precipitates from it on standing, as frequently happens in the case of human bile. And cholesterol stones of human origin introduced into the canine gall bladder are dissolved away.[7,8] In view of these facts the complete lack of cholesterol in our calculous material is scarcely surprising. Surprising it is, though, to find calcium carbonate the major constituent of dog stones. For though stones formed of the salt are the commonest variety in herbivora, they are seen in man only under very special circumstances; and one might suppose from the dietary habits of the dog that the same would be true of this species.

There exists much difference of opinion as to the source of the calcium in the carbonate stones of human beings. Not a few instances are on record of carbonate casts of inflamed ducts, resembling those which occur rather often in cattle. The general association of carbonate stones with marked inflammation of the passages has led to the view that the calcium has its source in inflammatory exudate. But within the tubing from some of our animals, and more especially within the cannulas taken, similar casts were present. They had formed by the union in a carbonate matrix of calculi primarily punctate (Fig. 10). Though a thin layer of organic débris was present with them, the calcium cannot in such instances have been the product of duct inflammation. For usually the ducts were

[23] Goodman, E. H., *Beitr. chem. Physiol. u. Path.*, 1907, ix, 91.

normal and the débris represented merely the accummulated products of normal desquamation from their surface. The fact has already been emphasized that the formation of carbonate stones went on, not upon but within, organic débris, as if induced by some secondary change or condition there. The question seems worth asking whether the active inflammation found in association with human carbonate stones may not tend to their formation by providing in organic débris a chemical nidus for deposition, while so interfering with the ducts that this débris is not voided as it would be under more ordinary conditions but retained to undergo changes preliminary to carbonate deposition. We shall present data which support such a view in a succeeding paper. Lichtwitz[17] has already given reasons for doubting the rôle of exudate in the production of carbonate stones, and Bacmeister[24] has shown that small amounts of calcium carbonate fall out of sterile human bile incubated *in vitro*, though not enough, he believes, to account for carbonate stones. A well marked precipitation of the substance usually takes place in sterile dog bile allowed to stand a few hours, as we have many times noted. Infection is in some way responsible for the carbonate stones met with at the postmortem table. But it may be so merely through its rôle as the principal agent whereby long standing inflammation of the biliary system is maintained in clinical instances.

Little is known of the conditions which lead to a deposition of calcium bilirubinate out of human bile. Bacmeister[25] has found bilirubinate stones in uninflamed and sterile gall bladders, but they are more frequent in the infected and damaged organ. In our dogs, calculi consisting predominantly of the substance were much less common than those of calcium carbonate, and they were usually restricted to the cannula and always to the upper reaches of the tubing. During the development of the calculi, carbonate was frequently deposited on bilirubinate stones—almost invariably, in fact, when the period of accretion was long—whereas the reverse process, bilirubinate on carbonate, was never observed despite the not infrequent secretion of a bile more concentrated than that put out in the days

[24] Bacmeister, A., *Beitr. path. Anat. u. allg. Path.*, 1908, xliv, 528.
[25] Bacmeister, A., *Ergebn. inn. Med.*, 1913, xi, 1.

immediately after intubation, when the bilirubinate core of the stones must have been laid down. There can be no doubt then that the conditions in the bile were, relatively speaking, unfavorable to bilirubinate deposition. It was noteworthy how often, just as in man, a central pigmented core of bilirubinate was present within calculi for the rest of another constitution. And as in the human creature, whole crops of such stones were sometimes present side by side. It was as if at some critical period in bile collection bilirubinate had come out of solution, providing here and there centers favorable to subsequent layering with another material,—serving in this respect just as foreign bodies (talc particles) were sometimes observed to serve, and as many sorts of foreign bodies have been noted to serve in man. The pronounced similarity in character of the stones of each crop or generation—for sometimes there was more than one—indicates that they were begun at one time and submitted to like growth conditions.

The punctate inception and development of the stones were to have been expected in the absence of preformed centers or nuclei, since deposition from slightly supersaturated solutions ordinarily takes the punctate form. But there existed special reasons therefor in many of the animals in the shape of the minute pigmented bodies about which stones tended to form. The source and composition of these bodies will be taken up in a paper to follow.

The presence in each calculus of an organic shadow or scaffold is in line with what is known of stone formation in general. Moritz,[26] Pfeipfer,[27] and Kleinschmidt[28] have noted that crystals falling out of dilute urine possess such shadows. The organic material of which they are composed comes down as an incidental to the deposition. Yet sometimes, as we have observed of carbonate stones forming in organic débris (Fig. 6, *a*, *b*, *d*, Fig. 7), the scaffold precedes the calculus, which latter is, so to speak, the result of petrifaction. Even in the case of bilirubinate calculi, the scaffolding sometimes grew faster than the stone because of the successive thin coatings with mucus that this latter received. The presence on the tube wall nearby of

[26] Moritz, cited by Bacmeister.[25]

[27] Pfeipfer, cited by Bacmeister.[25]

[28] Kleinschmidt, cited by Bacmeister.[25]

a similar mucous glaze rules out the possibility of a special influence of the stone to induce its deposition.

SUMMARY.

Gall stones frequently form in dogs intubated for the collection of bile under sterile conditions, in the absence of stasis and of gall bladder influence. The stones consist almost entirely of two substances—calcium carbonate and calcium bilirubinate—and they are remarkably uniform in character, as would follow from the limiting conditions of their development. They are not the result of bile loss, for similar ones may be recovered from the wall of glass tubes interpolated in ducts with intestinal connection undisturbed. The study of them has brought out evidence on the general problem of cholelithiasis. Some factors in their causation and that of gall stones as a class will be considered in succeeding papers.

EXPLANATION OF PLATES.

PLATE 12.

FIG. 1. Stones on the walls of the glass connections in a tube system,—from a dog sacrificed 24 days after intubation. The bile had been sterile. *A*, cannula containing calculi, some of which had become secondarily united and led to biliary obstruction. The absence of stones from about the entrance to the cannula should be noted. *B*, the curved tube that connected the arms of the rubber U. Here two "crops" of discrete calculi are to be seen. *C*, the T-tube from just above the collecting balloon. It contains small calculi only. × about 4½.

FIG. 2. Calculous deposit on the rubber tubing immediately below the glass connection in a U,—to show the discrete character of the stones and their greater frequency and larger size on the dependent side of the tubing and in the angle between rubber and glass. From the dog furnishing the material for Fig. 1. The tube has been laid open by a cut through the side that was uppermost during life of the animal. The area completely free from stones had been covered by the glass connection. × about 4½.

PLATE 13.

FIGS. 3 to 5. Calculi scraped from tubing through which sterile bile had been collected for 23 days. Figs. 3 and 4 show the stones by transmitted and reflected light respectively × 20; and Fig. 5 the shadows of them left after treatment with acid alcohol. × 36.

PLATE 14.

FIG. 6. A drawing of individual calculi from here and there on the wall of the cannula depicted in Fig. 1. *a*, *b*, *d*—carbonate calculi developing in the organic débris which had accumulated on the lower side of the tubing. *a* gives indications of the crystal form; and *d* has a pigmented center. *c*, stalactite forms of calcium bilirubinate from the upper side of the tubing. They are covered with a transparent skim of mucus. × 54.

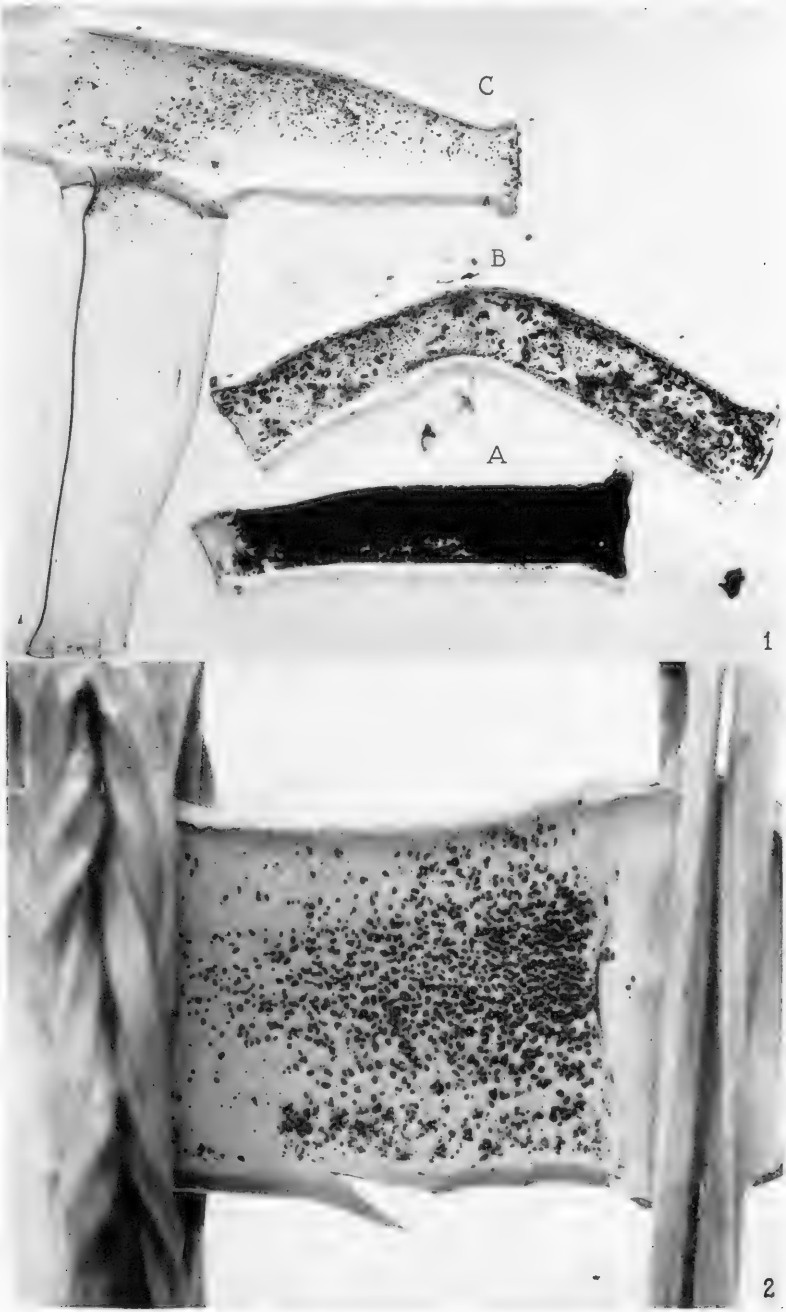
FIG. 7. Drawing of stones of two distinct types as they existed side by side on the walls of a cannula. The dark ones consisted of calcium bilirubinate, and those that were pale, and crystalline, of calcium carbonate. The latter were situate within small masses of organic débris. The dog had been intubated for 67 days, and the bile was infected after the 8th day. × 54.

PLATE 15.

FIGS. 8 and 9. Carbonate concretions from the wall of a glass tube that had been interpolated in the common duct of a healthy dog 35 days previously. The development of the stones by deposition in successive layers with retention of the crystalline form is well shown. × 77.

FIG. 10. Fragments of a stony cast of the inside of a cannula which had been connected with the common duct for 36 days during which time the bile remained sterile. An almost colorless deposit of calcium carbonate is to be seen on the large dark calculi. It had united these latter in a matrix. × 58.

FIG. 11. "Nuclei," some of them showing secondary deposition, from far down in the tube system of the same animal. All were intensely pigmented, but this feature is not evident in the photograph, owing to the use of a color filter which acted to minimize it. × 209.



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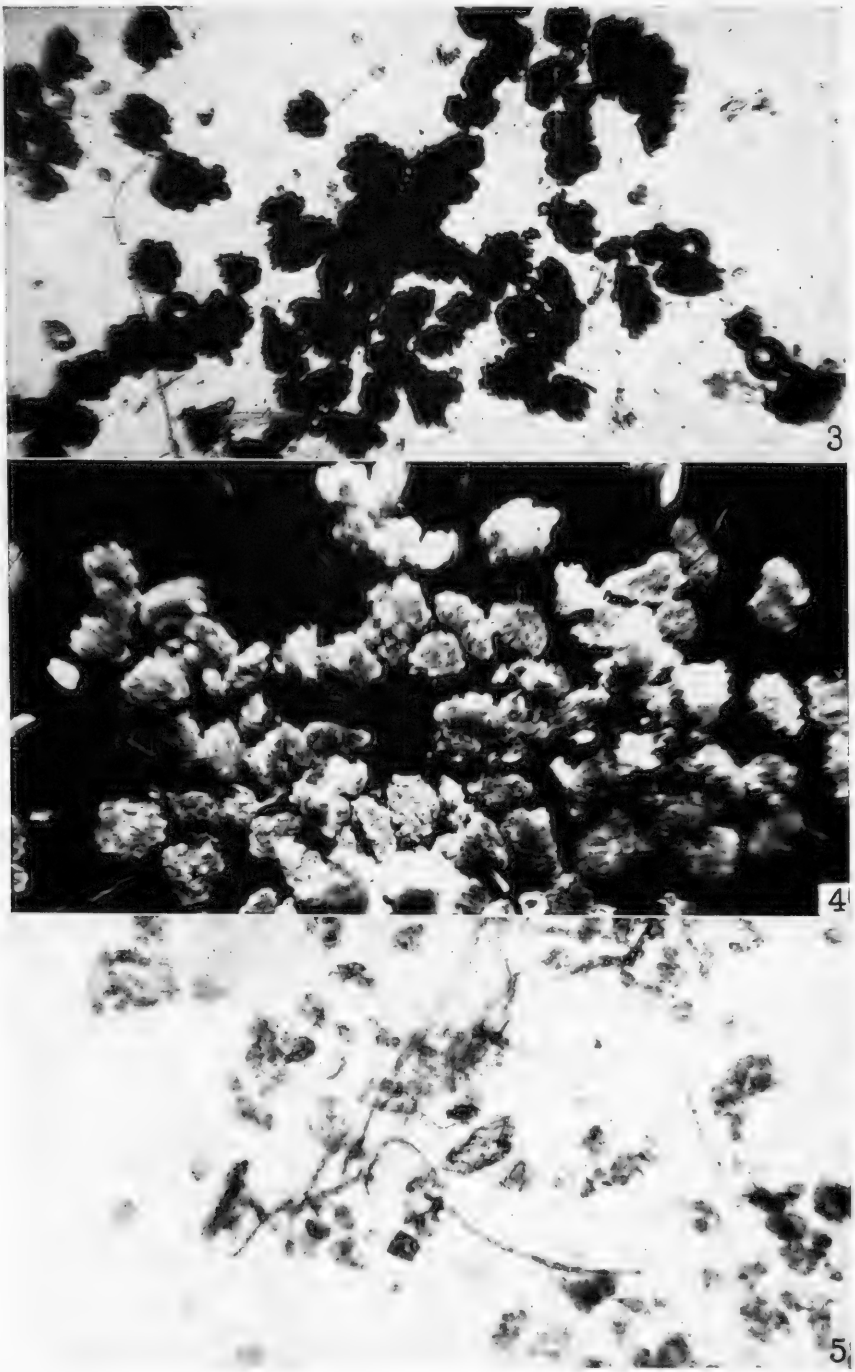

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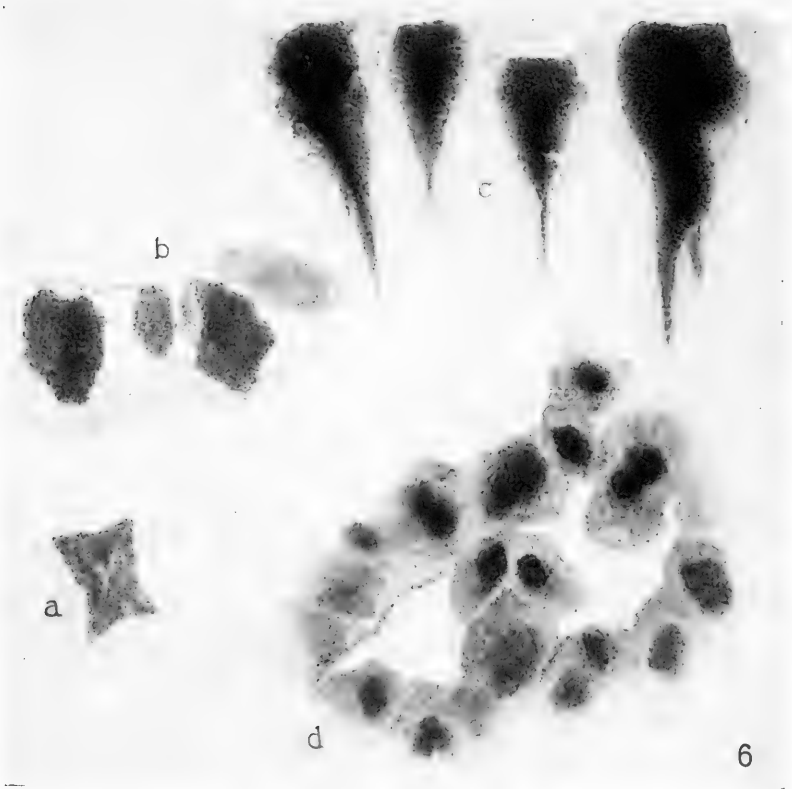

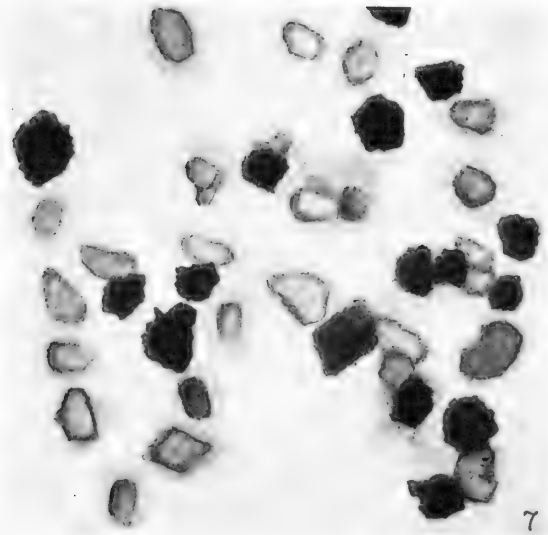

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OBSERVATIONS ON SOME CAUSES OF GALL STONE FORMATION.

II. On Certain Special Nuclei of Deposition in Experimental Cholelithiasis.

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Plates 16 to 19

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In a previous paper we have described gall stones developing in dogs under conditions so controlled that the complicating influences of infection, stasis, gall bladder activity, and inflammation of the bile passage may be ruled from account. The present communication embodies the results of a study of the early history of the stones with special reference to the rôle of preformed nuclei in their development.

Potential Centers of Stone Formation.

The calculi which form out of the liver bile of dogs permanently intubated for collection of the secretion consist almost wholly of two calcium salts—carbonate and bilirubinate[1]—in addition to the inevitable organic scaffolding or shadow. The conditions are relatively favorable to carbonate deposition. Only very occasionally does this appear to take place in the absence of a special center of deposition.[1] Sometimes a particle of talc from the wall of the rubber collecting tube serves the purpose of a nucleus, but more often the calculus forms in the midst of small masses of organic débris, or deposition of the salt occurs upon the surface of bilirubinate calculi. The formation of these latter is, by contrast, independent of organic débris, but often they contain minute, pigmented bodies, discrete

[1] Rous, P., McMaster, P. D., and Drury, D. R., *J. Exp. Med.*, 1924, xxxix, 77.

nuclei round which the stone seems to have been built up; while similar nuclei can be discovered not infrequently at the center of carbonate stones. As a whole, the findings indicate that the great majority of the calculi have their beginnings in preformed particles of one sort or another. The source and nature of such of these particles as have origin within the body will now be considered.

The importance of special centers of deposition for lithiasis has long been recognized; and many bizarre, and perhaps for that reason convincing, instances have been described. There is no need to enumerate the kinds of foreign bodies, including bacteria, reported in association with stones. Studies of the "strawberry gall bladder" have brought out the fact that small, rounded portions of the wall laden with cholesterol may be cast off into the bile; and it is supposed that upon these stone formation sometimes takes place.[2,3] Aufrecht[4] has advanced the opinion that pigmented particles, the product of disordered liver cells, may be set free into the bile and serve as the basis for stones; and Naunyn has suggested a rôle of "bile thrombi" in such relation.[5] From pathological gall bladders a variety of sediments may be obtained; and many believe that these have some connection with the development of stones. References to such sediments there have been from the time of Charcot, but no comprehensive study of them. Naunyn's brief surveys,[5,6] morphologic in their general character, and that of Lichtwitz[2] are perhaps the best on record. Aschoff[7] dismisses the subject with the statement that one can recover from the normal gall bladder all of the sediments (*Niederschläge*) which have been described as centers of crystallization. We have been unable to find the paper on the sediments by Torinoumi, which is mentioned by him.

Organic Débris in Dog Bile.

For observations upon the particulate matter in dog bile we have employed twelve dogs intubated for the purpose. The method and conditions of intubation have already been described.[8] A portion of the bile removed each day from the collecting balloon connected

[2] Lichtwitz, L., *Ergebn. inn. Med.*, 1914, xiii, 1.

[3] Boyd, W., *Brit. J. Surg.*, 1923, x, 337.

[4] Aufrecht, M., *Deutsch. Arch. klin. Med.*, 1919, cxxviii, 242.

[5] Naunyn, B., *Mitt. Grenzgeb. Med. u. Chir.*, 1921, xxxiii, 1.

[6] Naunyn, B., A treatise on cholelithiasis, Sydenham Society translation, London, 1896.

[7] Aschoff, L., *Klin. Woch.*, 1923, ii, 957.

[8] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 11.

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with the common duct has been centrifuged immediately and the sediment examined with the microscope and, when advisable, in divers other ways.

The balloons, like the remainder of the collecting system, were cleansed of soluble matter prior to use by boiling them in a weak solution of sodium bicarbonate, soaking in 2 to 5 per cent hydrochloric acid, and rinsing thoroughly. They were autoclaved twice in a large excess of distilled water. Often they were kept in the flasks of sterilization for some weeks prior to use. The bile which gradually assembled in them in the course of each 24 hours was protected from the air but had cooled somewhat, owing to its situation within a basket bound against the skin surface. The cooling was undoubtedly responsible for the major share of the precipitation observed, but the fact that one similar in kind went on within the animal was sufficiently shown by the character of the deposit upon the walls of the tubing inside of the body. To facilitate deposition in the tube system, bulbs about 1 cm. in diameter had been blown in the shanks of some of the cannulas (Fig. 17) and in some of the short glass tubes connecting the arms of the rubber U lying inside the abdominal cavity.

A heavy bile sediment was frequently obtained from animals with damaged livers. Afanassiew,[9] Brauer,[10] and others[11] have drawn attention to the presence in the secretion under such circumstances of coarse cylinders and of cells from the hepatic parenchyma. We have observed these after sublethal injury by chloroform, in company with red cells, leucocytes, and intact circlets or rings of desquamated epithelium from the ducts. Under such circumstances the bile may be red with blood and yet the animal be lively, have a good appetite, and in other ways appear healthy. Bile from normal livers, which had become infected during the course of the day-to-day collection, often contained numerous small mucous spheres, light green, and loosely grouped in wreaths or in bunches like grapes (Fig. 7). These were presumably derived from an abnormal duct mucosa, though no evidence of derangement was to be seen later in microscopic sections of the duct wall. Very occasionally the grouped spheres were present in sterile bile. In the secretion from an infected biliary tract, large numbers of small cylinders were sometimes to be found

[9] Afanassiew, M., *Z. klin. Med.*, 1883, vi, 281.

[10] Brauer, L., *Z. physiol. Chem.*, 1903–4, xl, 182.

[11] Haupt, H. G., *Arch. internat. pharmacod.*, 1903, xi, 155. Pilzecker, A., *Z. physiol. Chem.*, 1904, xli, 157.

(Figs. 5 and 6) which much resembled urinary casts both in size and appearance. Some consisted of brown, granular mucus, others were colorless and appeared homogeneous, while yet others were made up almost wholly of bacteria. They corresponded to the smaller type of cylinder described by Brauer. As a rule, no cells whatever were present in association with these elements which latter were obviously the result of a duct irritation less in degree than that causing desquamation. Much shorter casts, like small brown mucous plugs, were found on one occasion in the uninfected, deeply pigmented bile secreted after intravenous injections of dog hemoglobin.

Hemoglobin injections caused a thickening of the bile with mucus, as many previous workers have noted. In the sediment this had the appearance of brown filaments or ill defined, granular clumps. Small, flat, shreddy masses or sheets of mucus were not infrequent in "normal" biles, when the cannula had caused local irritation of the duct by pressure.

The sterile bile from healthy ducts was often almost water-thin and contained, even on cooling, practically no mucus that could be thrown down with the centrifuge,—far less than the ordinary nubecula of urine. But a well defined sediment there usually was, one of more or less pigmented and imperfect calcium carbonate crystals. And we have repeatedly observed in the sediment early stages in the formation of carbonate stones identical in type with others, often much larger, recovered at autopsy from the tubing within the animal. They tended to develop about nuclei of special constitution. But before the nature of these latter is considered the fact should be emphasized that none of the organic débris just described was ever observed to act as a center for stone formation. In a previous paper[1] the frequent situation of carbonate stones within the layer of organic material sometimes coating the tube system has been emphasized, and the opinion has been expressed that the association is such as to indicate that some secondary change in the material is responsible for the inception of the stones. The fact that the material, when fresh, as in the bile sediment, does not induce carbonate deposition despite a tendency for the salt to come out of solution adds further support to this view.

Nuclei of Deposition.

The bile of the first 24 or 48 hours after the operation whereby intubation is effected, though often greatly concentrated, at least in some substances,[12] yields as a rule no sediment other than mucus, which for the time being is increased in amount. That of the 2nd or 3rd day contains a slight brown, or greenish brown, dust-like powder, less than $\frac{1}{100}$ cc. to a 15 cc. bile specimen. Under the microscope this dust has the appearance of minute, highly refractile spheres (Figs. 9 and 10), some separate, some partially fused into diplococcoid (Fig. 8) or dumb-bell forms, or into irregular concretions or even into elongate "worms." These nuclei, as we shall term them, are much heavier than water and bear washing with it in the centrifuge. They are brilliant with brown or yellow-brown pigment but can be decolorized by a few hours' incubation with commercial hydrogen peroxide that has been rendered neutral with sodium hydrate; and they are then seen to be doubly refractile. They may persist in the bile, though in diminished quantity, until about the 6th day after operation, but usually are not met thereafter unless the liver is injured in some way, when they may again appear for a greater or less period. They serve as centers for calcium carbonate deposition (Figs. 8, 11, 12, and 16), and in some animals only those first found after intubation are free from crystals of the salt or a concentric layering with it. The contrast between the well-nigh colorless carbonate and the deeply pigmented nucleus upon which it has come down is often a striking one.

Needless to say, the collecting balloon was emptied as completely as possible each day, of sediment as well as of bile. Thus stone formation within it was periodically interrupted, and only microscopic calculi could be expected in the sediment.

Before discussing the nuclei in detail, it will be well to meet the question whether they and the minute stones recovered from the bile of the balloon had any real connection with the calculi recovered from the tubes within the animal. Proof of the development of such nuclei *in vivo* was forthcoming in the instance of an animal the sterile bile from which contained mucous casts obviously derived from small ducts, with brown nuclei along their central axis identical

with those we have just described. Other animals yielding nuclei into the balloon showed them at autopsy lodged here and there on the walls of the tube system. Often when stone formation had gone on for a considerable time, the large calculi sessile on the walls of the cannula and of the few centimeters of tubing nearest the bile source showed nuclei at their center (Figs. 13 and 18); while, further down, small nucleated stones were met, identical with those sometimes present in a 24 hour specimen from the balloon; and lower yet, nuclei were recovered in the free state from the tube wall. Free nuclei were indeed frequently present on the tubing of animals which failed to develop stones, whence it follows that they did not inevitably induce lithiasis.

Nature of the Nuclei.

What was the character and derivation of the nuclei?

They failed to take the stains for neutral fats (Sudan III, Scharlach R), but with Nile blue sulfate were colored a deep blue like the fatty acids. They did not consist of myelin, since they proved insoluble in alcohol; nor did they contain cholesterol as was sufficiently shown by their resistance to ether, to acetone, and to boiling chloroform, and by the further fact that chloroform extracts failed to give the Liebermann-Burchard reaction. They did not stain by Gram. Heat fixed them on the slide but they retained shape and remained anisotropic despite it. Dilute hydrochloric acid (10 per cent) in water deprived them of all refractility; they became somewhat smaller and the irregular forms became spherical. Sometimes they effervesced on such treatment and again not, but regularly they changed color, turning gradually green-black or deep blue. It was this deep color and the persistence of the forms as well-defined shadows which enabled us to distinguish them at the centers of large calculi softened with hydrochloric acid and flattened by pressure.

From the reactions as given, it is evident that calcium carbonate must have been one constituent of the nuclei. Their doubly refractile character may well have been referable to its presence. Yet they cannot have consisted only of carbonate tinted with bilirubin. For much of their substance survived the treatment with acid. The pigment did not come away from them in chloroform even on boiling, though after subjection to acid it passed into this solvent of bilirubin and then gave the Gmelin reaction. Calcium bilirubinate, the substance concerned in the more darkly colored calculi within the tube system,[1] behaves in this way. Stones composed in great part of it appear soft in comparison with carbonate stones, fracturing upon pressure into rosettes of fragments with rounded angles.[1] This was the case too with the nuclei (Fig. 10). A well defined shadow

or scaffolding of elastic mucus was brought out by treatment of them with acid alcohol, in which their other constituents dissolved.

Altogether, it would seem that two substances besides the organic shadow were the main ingredients of the nuclei, calcium carbonate and calcium bilirubinate, this latter being the more important as distinguishing them from ordinary forms of carbonate precipitate in the bile. Their appearance and fracture pattern suggest attempts at crystallization.

The nuclei, as already stated, were recovered from the bile of healthy animals only during a brief period after the operation to insert the collecting apparatus. But they were frequently met with at other times when the liver was purposely damaged.

Thus, for example, they were seen in association with delayed chloroform poisoning, as also when metatoluylenediamine had been given by mouth. Concurrent observations on the incidence of nuclei and the pH of the bile were frequently made with the potentiometer, but no relationship between the two could be made out. Nor was the total calcium content of the bile, as obtained by ashing, different when they were present than when they were not. Ether anesthesia of several hours' duration did not cause them to appear. Intravenous injections of a concentrated calcium chloride solution were sometimes followed by a "shower" of them, and again not; and they appeared sometimes immediately after the relief of total obstruction of 24 to 48 hours' duration. Increased concentration of the bile consequent on prolonged fasting[12] did not cause their occurrence even when the change was great, as in one instance in which the secretion diminished from more than 150 cc. in a 24 hour period to between 11 and 12 cc. on each of 2 successive days with a reciprocal increase in the concentration of bilirubin.

Carbonate Deposition.

The stones forming out of calcium bilirubinate in the intubated animals were always restricted to the few centimeters of tubing nearest the bile source.[1] From this it would appear that there was relatively little of the material available for calculi. The state of affairs as concerns calcium carbonate was wholly different. Crystals of the salt (Fig. 14), not infrequently a copious sediment of them (Fig. 15), were in most instances present in the balloon specimens from a day or so after operation until the observations were terminated, weeks

[12] McMaster, P. D., Broun, G. O., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 395.

or months later. The deposition of carbonate on the walls of the intraperitoneal tubing during the same period was comparatively slight, and took the form, not of a sediment but of discrete calculi. It follows that the precipitation in the balloon must be ascribed for the most part to extraneous factors. There can be no doubt that cooling was chief amongst these. Chilling the fluid in the ice box frequently brought on additional precipitation.

The crystals were sometimes perfect and almost colorless, and again, when they had come down out of a dark bile, deep yellow and poorly formed. Often their angles were blunted, or, when they were present in aggregates, these had undergone a rounding, evidently because a portion of the material had dissolved again. In this way spherical nuclei of the sort we have just described were sometimes simulated. But the substance of such pseudonuclei showed planes of cleavage instead of being homogeneous, and on treatment with acid they effervesced and vanished wholly save for a lightly tinted shadow. Occasionally forms were noted which can only be considered as intermediate between true nuclei and carbonate crystals. From a study of them one gains the impression that they arose through the combination in a different proportion of the same materials found in the true nuclei, that is to say crystalline carbonate and brown, amorphous bilirubinate. Many of the stones described in our previous paper[1] were so constituted.

Concentric deposition is a marked characteristic of the carbonate stones of human beings. The minute stones recovered from the balloon specimens of our dogs usually exhibited this characteristic pronouncedly (Fig. 19). Most of them were slightly pigmented, appearing of a brilliant green or yellow under the microscope.

The Rôle of Bile Thrombi.

When the liver had been damaged by methods that caused jaundice, elements closely resembling the so called bile thrombi that are present in the hepatic tissue under such circumstances appeared in the bile itself (Figs. 1, 2, 3, and 4). The question arose of their identity as also that of whether the nuclei just considered might not, though differing in aspect from bile thrombi, be composed of the same materials.

Heinrichsdorff[13] has employed microchemical methods to study the bile thrombi in formalin-fixed or frozen sections of the human liver. He bleached them with hydrogen peroxide and found that they were not anisotropic, though highly refractile. They exhibited the various forms which arise by the partial merging of a greater or less number of spheres, and they were almost completely destroyed by hydrochloric acid, suggesting, as the author remarks, that they contained calcium, though he was unable to demonstrate its presence. They were Gram-positive and became deep blue when submitted to Nile blue sulfate, but were only dubiously tinted by Sudan III. They gave some of the reactions for lipoids. Those from a case of hepatic carcinomatosis differed in minor details from such as were the result of ordinary biliary obstruction.

We have compared the thrombi of icteric dog livers with elements morphologically similar recovered from the bile of the same animals, and with the little nuclei occurring in the bile after the intubation of healthy dogs.

Icterus was produced by the oral administration of toluylenediamine or by gradual biliary obstruction or prolonged chloroform anesthesia. The thrombi were freed from the liver tissue by scraping the cut surface with a knife wet with water or salt solution, suspending the pulp thus obtained in one of these fluids, and filtering through several layers of gauze. There resulted a suspension of individual cells and thrombi. The latter could now be examined directly, or they could be rid of their association with tissue elements through the action of trypsin in 2.5 per cent solution rendered slightly alkaline with sodium bicarbonate. The thrombi alone survived the tryptic digestion and after a few hours of it at 37°C., could be recovered in enormous number by centrifugation. In one exceptional instance of a few scattered thrombi recovered from a liver shortly after chloroform poisoning, these were destroyed at the same time as the cells.

The thrombi were found to answer closely the description of those studied by Heinrichsdorff. The small ones were spherical or oblong, and the larger ones irregular, or even twig-like, and occasionally forked. They were obviously formed by the more or less perfect fusion of small spheres from within the liver cells, which spheres survived the treatment with trypsin as well as did the thrombi. The material composing them was homogeneous in appearance, highly refractile, and ranged from yellow to dark brown. They were readily bleached with peroxide and were not anisotropic, thus differing essentially from the nuclei of the bile sediment. They were much lighter than the nuclei, having approximately the same specific gravity as the liver tissue. They were Gram-positive, stained faintly to darkly blue with Nile blue sulfate, but were not colored at all with the

[13] Heinrichsdorff, *Berl. klin. Woch.*, 1920, lvii, 1217; *Centr. allg. Path. u. path. Anat.*, 1922, xxxii, 314.

stains for neutral fats; and they were unaffected by water, glycerol, ether, ethyl alcohol, chloroform, and acetone. They could be fixed on the slide by heat without loss of shape. They gave Gmelin's reaction. On standing in water in an open vessel, they gradually turned from brown or yellow to green, as would have been the case had the pigment associated with them been bilirubin as such. The pigment of the nuclei in the bile sediment remained, by contrast, unchanged even after several days in water, an additional reason beyond those already supplied for supposing it to exist in combined form (calcium bilirubinate). Dilute hydrochloric acid caused the thrombi usually, though not always, to lose their refractility together with most of their substance, a pigmented shadow alone remaining.

From this description it will be evident that in many of their characters the thrombi of jaundiced livers resemble the nuclei found in bile sediment, but that in others, and these essential ones, they differ therefrom. The elements in the bile from damaged livers which looked like liver thrombi gave identical reactions with those for these latter, as just enumerated. There is good reason to suppose them to be composed of the same materials. But that they came down as such through the narrow and tortuous bile radicals from an original situation within the lobule is open to doubt. No deposition of carbonate upon them was ever observed.

Nuclei in Human Bile.

In continuation of the work, it has seemed worth while to examine the sediment of human bile, and we have been enabled so to do through the generous cooperation of Doctor Allen Whipple of the College of Physicians and Surgeons of Columbia University. Several fistula specimens were procured from patients recently relieved of obstruction and with rapidly diminishing jaundice. All appeared highly dilute, in keeping with what has been observed of dog bile under like conditions of readjustment.[12] None yielded sediment other than an occasional cell. The nine consecutive cases furnishing gall bladder specimens were a diverse lot, comprising two instances of common duct obstruction by carcinoma of the pancreas, one of a definite cholecystitis without stones, two of dubious cholecystitis, and four with stones and a chronic mild inflammation of the gall bladder. No significant sediments were encountered except in this last group, only flakes of more or less stained mucus and a few

desquamtaed cells. But in the very dark, thick biles of three of the four cases with stones, there was present in addition to these latter a sediment consisting of mucous flakes and particles, desquamated cells, cholesterol plates, and minute concentric concretions, or spheroliths, consisting of calcium carbonate (Figs. 20, 21, and 22); while, in one of the three, early stages in the development of cholesterol stones about such concretions were also found (Figs. 23 and 24) and in two of them the concretions were demonstrable within well formed calculi (Figs. 25 and 26). In the third case they were not sought there. Both through their character and relationship to stone formation as thus attested, the little spheroliths bore a striking resemblance to the nuclei we have encountered in dogs.

The carbonate concretions were as a rule spherical or boat-shaped (Figs. 21 and 22), more rarely diplococcoid or lemon-shaped, or with rays like a blunt star (Fig. 20), or even worm-shaped through the partial fusion of several spheres. They were yellow, green, brown, or colorless, and showed sharp-cut concentric zones, often further demarcated by pigment variations. Most, but not all, were brilliantly anisotropic. They resisted acetone, failed to stain with Scharlach R, but were rendered light to dark blue by Nile blue sulfate. They could be fixed on the slide by heat which, for the rest, seemed not to change them. Under pressure they broke along both radial and concentric lines. They effervesced actively when treated, after washing in water, with a dilute watery solution of hydrochloric acid applied with a micro-pipette, and the greater part of their substance rapidly dissolved, leaving a more or less pigmented shadow having the original shape (Fig. 26). That these carbonate spheroliths were not an *in vitro* product was sufficiently proven by their presence deep within some of the smaller calculi associated with them (Fig. 26). The primary stones were not examined, unfortunately, but in both the instances in which secondary stones were studied with reference to the point such inclusions were found. The stones consisted predominantly of cholesterol in these cases but differed in that those of one dissolved in acetone completely save for a thin mucous shadow and the contained spheroliths, whereas those of the other underwent only a surface disintegration in this solvent but when subjected to weak hydrochloric acid effervesced and fell apart into a multitude of cholesterol plates (Fig. 26). Evidently these latter had been held together in a matrix of calcium carbonate. Fragments of a large radial cholesterol calculus were present in the bile of the case first mentioned; and even before acetone treatment it was evident that these contained carbonate spheroliths. Afterwards, in the mucous shadow of them, the spheroliths stood forth strikingly, often several to a stone fragment. Curiously enough, they

interfered almost not at all with the radial structure. The radial lines parted about them and joined again like threads of silk about bullets placed within the skein. Obviously the spheroliths were only accidental inclusions. Far different was the condition of affairs within certain other small calculi from the same animal. These showed a concentric structure, with sometimes a pigmented, earthy looking core (Fig. 25), though cholesterol predominated in them; and near their center one or more spheroliths were usually to be found. It cannot be said on such evidence that the stones had been built up about the spheroliths. But among the free bodies of this latter sort were to be noted some that had a thick surface layer of cholesterol plates, or of stained organic material (Figs. 23 and 24). Such beginnings of stone formation were not encountered in another bladder specimen searched for them; the stones, though small, were all fully formed. But within them after acid treatment the discrete shadows of spheroliths could be discerned. The bile of both cases was sterile on culture (aerobic, anaerobic), and microscopically as well. In a third case, in which the spheroliths existed as such, the secretion proved to be infected.

The spheroliths are probably the same that Naunyn terms calcium bodies and describes as highly refractile, smooth and spherical, rugose, or occasionally shaped like "morning stars." We have gone into details concerning them because they have received but scant notice in the past; while furthermore their relationship to the genesis of secondary stones seems not to have been noted. It is our impression from the few instances studied, as from the literature on the sources of calcium in human bile, that, unlike the nuclei we have recovered from dogs, they do not arise out of liver bile as such but from the contents of abnormal gall bladders. A large material should be studied with relation to their presence and their rôle in stone formation.

DISCUSSION.

There has been much debate among students of cholelithiasis over the share therein of organic débris. Naunyn[5] describes soft organic lumps upon which he believes that calculi are built up; and Bacmeister[14] holds that the common faceted stones, which are, as he remarks, the great majority of gall stones, form about desquamated epithelial cells, cell nuclei, bacteria, or mucus, by a deposition of calcium as carbonate or in organic combination. These are but two

[14] Bacmeister, A., *Beitr. path. Anat. u. allg. Path.*, 1908, xliv, 528.

among many opinions. Our observations make clear the fact that, in the dog at least, organic débris of the sorts mentioned is frequently voided in quantity under circumstances of liver or duct derangement, but that no deposition from the bile takes place thereon despite a well defined tendency for the calcium carbonate in the fluid —the principal constituent of calculi—to come out of solution. The deposits of calcium carbonate in spherolith form, and of cholesterol crystals and amorphous calcium bilirubinate, which we have encountered in the bile from human instances of cholelithiasis, have likewise proved to be independent of the numerous flakes and particles of organic débris also present in the sediment. On the other hand, calcium carbonate is, in the dog, frequently laid down within old accumulations of such débris presumably as a consequence of secondary changes or conditions therein;[1] while in man carbonate calculi have a notable association with severe inflammation of the biliary tract such as would favor accumulations of the sort. The distinction thus brought out is an important one. Organic material cannot accumulate in the bile channels except when they are for some reason poorly evacuated. Lessened motility of the ducts combined with an unusual supply of such material will of course have such result. And it may be significant that many of the successful attempts to induce stone formation in animals have involved procedures whereby both of these conditions are fulfilled (Gilbert and Fournier,[15] Mignot,[16] Ehret and Stolz,[17] and others). True, the authors of the experiments have considered infection to be the prime agent in the cholelithiasis; and though they lay emphasis on the importance of lessened motility, it is as furthering infection. But our work leaves no doubt that in the dog, at least, carbonate stones will form out of bile in the absence of infection; while that the substance can on occasion come out of sterile human bile in spherolith form is sufficiently shown by the findings in the few instances of cholelithiasis we have here described. Bacmeister[18] has noted the precipitation of small amounts of calcium carbonate out of sterilized human bile incubated

[15] Gilbert, A., and Fournier, L., *Compt. rend. Soc. biol.*, 1897, iv, 936.
[16] Mignot, R., *Arch. gén. méd.*, 1898, ii, 129.
[17] Ehret, H., and Stolz, A., *Berl. klin. Woch.*, 1902, xxxix, 13.
[18] Bacmeister, A., *Münch. med. Woch.*, 1908, lv, 211.

in vitro, and both he and Aschoff have questioned whether carbonate stones may not sometimes form in the absence of infection. Proof in the matter has heretofore been lacking.

In a previous paper we have advanced the view that the infection so often encountered in association with human stones consisting of calcium carbonate may act to cause them not by effecting changes in the bile favorable to precipitation,[19] as some have supposed, nor by eliciting though inflammation an exudate rich in calcium, as others have thought, but merely by setting up and maintaining such a pathological condition as would tend to further the accumulation of organic débris and its retention long enough for certain changes to take place which are a preliminary to carbonate deposition. The physicochemical circumstances determining the calcification itself may well be akin to those through which calcium salts come to be laid down in necrotic foci elsewhere in the body, though with this difference, of course, as concerns the biliary calculi of the dog, that no phosphate is deposited.

The activities of the gall bladder to alter and concentrate the bile and to elaborate calcium carbonate have not thus far been referred to because carbonate stones can form without their aid, while a recognition of them in our discussion greatly complicates the issue. It stands to reason that gall bladder conditions and activities must be of the greatest importance for certain clinical instances of lithiasis. They may well have been a prime factor in the causation of the calcium spheroliths we have described, which latter were recovered from thick, dark, bile specimens that had obviously undergone concentration and an addition of mucus.

Naunyn's query[5] as to whether "bile thrombi" coming down out of the liver may act as nuclei for calculus formation can be answered in the negative as concerns the experimental lithiasis with which we have had to do. The particles of what would appear to be thrombus material present in the bile from damaged livers were never observed to function as centers of deposition, unlike the minute pigmented bodies, or nuclei, consisting of calcium bilirubinate and carbonate that were often associated with them. These latter were a frequent precursor of lithiasis. That they were not preformed in

[19] Galippe, V., *Compt. rend. Soc. biol.*, 1886, xxxvii, 116.

the liver seems certain; for they were never to be seen in scrapings of the tissue. Their appearance suggests that they represent attempts at crystallization out of an impure solution. Bacmeister[18] has pictured spherical bodies not unlike them in appearance, though formed of cholesterol, which sometimes come down out of human bile long incubated *in vitro* under sterile conditions; and Bolt and Heeres[20] have reported that spheres of cholesterol come down out of the "bile" elaborated by frog livers perfused with salt solution. Our observations link such findings with actual body processes. The sequence of events as witnessed in the intubated dog is somewhat as follows: Upon damage to the liver, an abnormal bile is elaborated out of which the calcium nuclei fall, and some of them lodge on the walls of the tube system there serving as centers of deposition of calcium salts from the later, more normal, secretion. That the nuclei actually induce deposition from bile out of which it might not otherwise occur remains to be proven. In some instances, certainly, they persist for a long while in the tube system and no stones develop. But in the great majority of instances their presence is soon followed by lithiasis. The stones of such of our animals as were subjected to the single disturbance involved in intubation and were sacrificed early differed from those of others killed later only in that the calculous deposit about the nuclei was in general less considerable. Both sets of stones appeared to date back to the same period of inception, namely to the days immediately after operation when nuclei came down out of the bile. The occasional presence of several crops or generations of stones can be accounted for by the occurrence at subsequent times of liver damage as result of which showers of nuclei appeared.

The frequent presence in the human gall bladder of large numbers of calculi of practically identical size and character has led to a general recognition of the importance in such instances of critical periods of stone inception and formation. And the existence of nuclei of special composition at the center of the great majority of human stones has been frequently commented upon. Lichtwitz[2] has emphasized the point that the stone nucleus is the same or nearly the same in all human calculi, while asking whether this nucleus

[20] Bolt, N. A., and Heeres, P. A., *Arch. ges. Physiol.*, 1922, cxciii, 449.

forms on an inflammatory or non-inflammatory basis. It is possible that as in the dog, showers of nuclei sometimes come down out of the bile of pathological conditions. Our few observations on the occurrence of carbonate spheroliths in the human gall bladder and their relationship to the genesis of secondary stones are merely suggestive in this connection.

The relation of the calcium spheroliths of human and canine bile to the calcospherites sometimes encountered in the tissues is a matter upon which one can at the moment merely speculate.

The fact has already been stressed that stone formation is no inevitable consequence of the presence in dog bile of nuclei of the sort just discussed. They were sometimes recovered in quantity as such from the tubing of animals the bile of which had yielded them only once, and this weeks previously, in the postoperative period. There were exceptional animals which secreted bile that never throughout a long period of observation gave a precipitate even after 24 hours in the ice box. The food, treatment, and general condition of these dogs did not differ from that of the generality, bile from which gave day after day a marked carbonate deposit. One dog of a previous series had at autopsy good sized carbonate stones that had formed in the absence both of nuclei and of cellular débris.[1] All of which is to say that there are peculiarities of the individual which make decisively for or against carbonate lithiasis.

In our previous paper the question was raised why the stones of intubated dogs are never found in the ducts but are always limited to the foreign tubing; and a provisional answer was offered that duct motility, with the cleansing and possibly antagonistic action of the secretion from the mucosa, must be responsible. The question comes up more insistently in connection with the normal accumulation of bile in the gall bladder. Were the viscus a mere collecting bag for the highly unstable secretion as it comes from the liver, cholelithiasis could not but be one of the commonest of canine afflictions, especially since the bladder often contains a layer of old organic débris such as conduces to carbonate deposition. That stones are almost never met with in the unintubated dog argues for some special physiological safeguard against their formation. The aspect thus presented of the problem of cholelithiasis will be dealt with in a subsequent communication.

SUMMARY.

A day-to-day study has been made of the sediment in the sterile liver bile of intubated dogs. There exists a marked tendency for calcium carbonate to be deposited therefrom. After hepatic or duct injury a great deal of organic débris of various sorts may be present in the bile, but it never causes deposition out of the fluid save when it accumulates in quantity on the tube wall. Particles of the material of which "bile thrombi" are composed may be found in the bile when the liver has been appropriately damaged, but these fail to act as centers of stone formation. On the other hand, there are to be found in the secretion after many sorts of hepatic injury little nuclei which undoubtedly serve in this way. These nuclei consist of a mixture of calcium bilirubinate and carbonate with an organic shadow or scaffolding. They do not occur in normal bile, but, coming down on special occasions, prove favorable to deposition out of the secretion elaborated at later periods. We have found nuclei strikingly similar in their relationship to stone formation, but consisting almost wholly of carbonate, in the sterile bile from human gall bladders which contained large calculi. Many of them were encountered in a free state, and others with layers of cholesterol and organic matter upon their surface, while others yet were recognizable deep within matured stones.

The factors concerned in the genesis of gall stones consisting of calcium carbonate have been reviewed at some length in this paper and the preceding one. The evidence we have collected supports the view that the development of carbonate stones in human beings as well as in the dog may be a consequence, not of changes in the bile brought about by microorganisms, nor of the elaboration of an inflammatory exduate rich in calcium salts, but merely of inflammation such as leads to lessened motility of the duct system with the accumulation of organic débris. The fact that infection is almost the sole agent whereby such inflammation is set up and maintained in clinical instances had led too often to the conclusion that it serves as the essential agent in the process of calcification.

The present findings taken with those described in a preceding paper suffice for an understanding of the immediate history of the gall stones which develop in intubated dogs. But the factor of in-

dividual differences, as yet undefined in nature, bulks large in the problem of the lithiasis, as does that of the local physiological safeguards against it.

EXPLANATION OF PLATES.

PLATE 16.

FIGS. 1 and 2. Bile thrombi from a liver that had become icteric as the result of obstruction of the common duct. These were photographed in fresh preparations made by scraping the cut surface of the liver, suspending the pulp in water, and centrifuging. × 730.

FIGS. 3 and 4. Thrombus-like forms obtained from the bile of an intubated dog on the 6th day of chloroform poisoning. The animal was jaundiced. The bile was sterile. × 730.

FIG. 5. A brown, mucinous cast from the infected bile of an intubated dog. × 730.

FIG. 6. A bacterial cast from the same specimen. × 730.

FIG. 7. Mucous globules in wreath form from an infected bile. They were bright green. × 730.

FIG. 8. Secondary deposition of an almost colorless layer of calcium carbonate on a diplococcoid calcium nucleus of yellow hue. The layer shows some radial striæ. The specimen was obtained on the 6th day after operation, from the same animal that yielded the material of Figs. 6, 7 and 8 (*q.v.*). × 730.

PLATE 17.

FIG. 9. Deep brown, calcium nuclei recovered from bile secreted on the day after intubation of the animal. Their variations in shape and high refractility should be noted. × 730.

FIG. 10. Fracture forms of the same nuclei = rosettes. × 730.

FIGS. 11 and 12. Deposits of almost colorless carbonate crystals upon pigmented calcium nuclei like those just figured. There are many free crystals as well. The preparation shown in Fig. 11, which is magnified 600 diameters, came from the bile of the same animal furnishing those of Figs. 9 and 10. It was obtained on the 2nd day after operation. Fig. 12 is from the bile of another dog, collected 4 days after operation. × 730.

FIG. 13. Demonstration of calcium nuclei lying within the small gall stones found at post mortem on the tube wall in a dog sacrificed 14 days after intubation. The animal had previously furnished the material of Fig. 12. The stones came from just below the cannula. They are treated with weak hydrochloric acid which dissolved the layers of calcium carbonate surrounding the nuclei and rendered the shadows of the latter more distinct. × 730.

FIG. 14. Sediment of coarse carbonate crystals from the later bile of the dog furnishing the material of Figs. 9, 10, and 11. The specimen pictured was procured on the 4th day. × 600.

PLATE 18.

FIG. 15. Carbonate sediment in the later bile of the dog furnishing the material of Figs. 12 and 13; specimen of the 6th day. About one-half natural size.

FIG. 16. A layer of crystalline carbonate enclosing a group of calcium nuclei. There was in this instance an especially brilliant color contrast, scarcely visible in the reproduction. × 730.

FIG. 17. Cannula with interpolated bulb, as removed at autopsy on the 14th day after intubation from the dog furnishing the material of Figs. 12, 13, and 14. The punctate distribution, dark color, and angular shape of the calculi should be noted. Nearly all of them were on the side of the cannula that was lowest during life. There are many more in the bulb than elsewhere but they are smaller there, doubtless because of the fact that they soon became overlaid with mucus. The duct end of the cannula is free from stones. × 5.

FIG. 18. A heavily pigmented carbonate bilirubinate calculus of the sort pictured in Fig. 17, after treatment with hydrochloric acid. The included nuclei are well shown. The liver had been severely damaged with chloroform 2 days prior to death, which may account for the presence of peripheral nuclei. × 450.

FIG. 19. Minute concretions of calcium carbonate from a sterile specimen of bile obtained on the 19th day of intubation. The concentric deposition is evident. × 730.

PLATE 19.

FIGS. 20 and 21. Minute concretions consisting almost wholly of calcium carbonate, as observed in the gall bladder bile of a patient with mild cholecystitis and many large gall stones. The bile was sterile. × 150.

FIG. 20 shows a "morning star" form, as also some cholesterol plates and organic débris. Fig. 21 shows only calcium spheroliths, but these with concentric zone varied by color differences. All are much larger than the nuclei found in dog bile (Figs. 9 and 10). × 150.

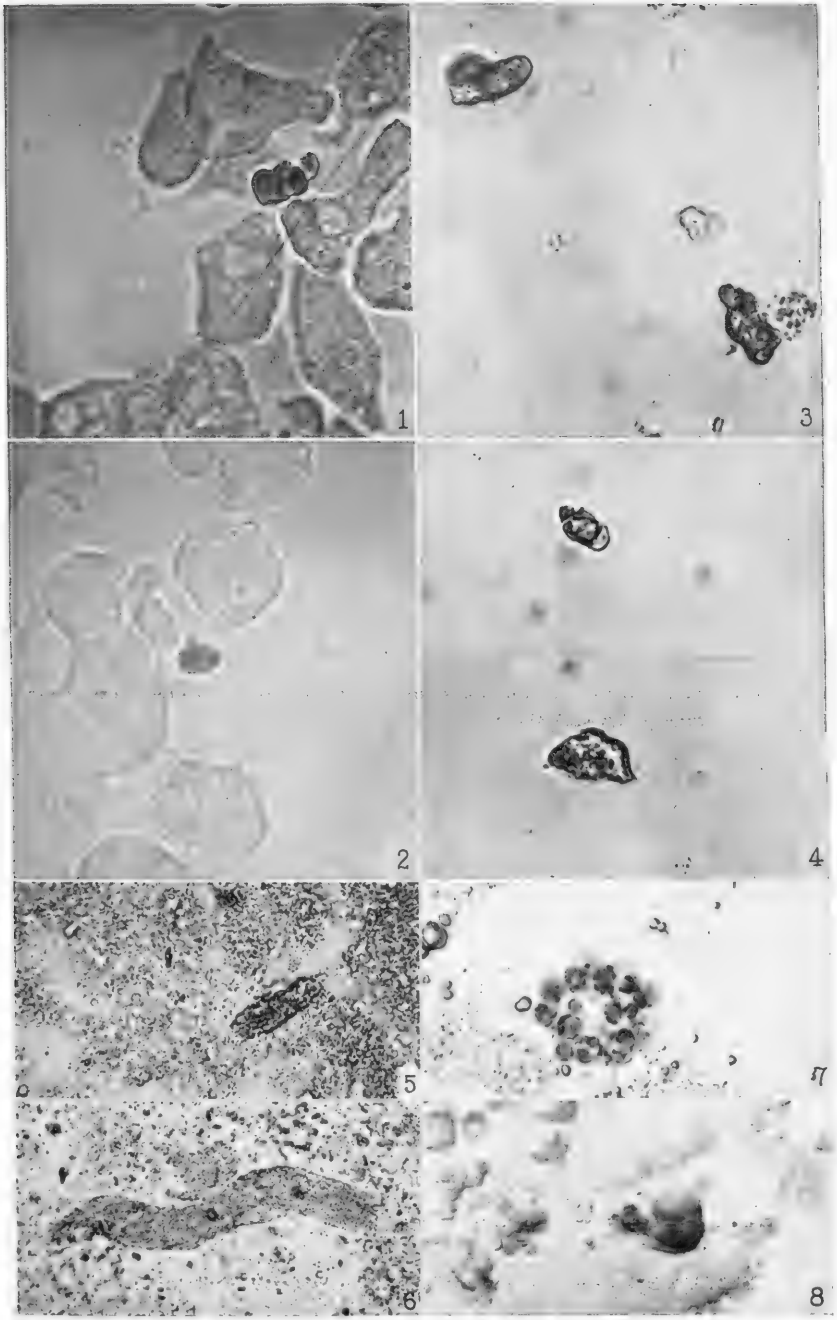
FIG. 22. Carbonate concretions from the bladder bile of another patient with cholelithiasis and mild chronic cholecystitis. In addition to spheroliths, boat forms and forms of lemon shape were present. The bile was infected. × 150.

FIG. 23. A carbonate spherolith from the bladder bile of yet a third patient with cholelithiasis. The bile was uninfected. A rind of stained organic material covers the little concretion. Owing to the use of a color filter to bring out the shape of the nucleus, this latter appears far darker than it really was, and the covering layer much lighter. × 290.

FIG. 24. Another spherolith from the same bile specimen, upon which cholesterol has been thickly deposited. Plates of the substance can be seen here and there, and those between the observer and the spherolith make it seem irregularly striated; but the bulk of the cholesterol deposit is only evident in the photograph as a highly refractile zone. There is much free organic débris round about, and at one side a smaller spherolith. × 290.

FIG. 25. Small calculus from the bladder specimen furnishing the material of Figs. 20 and 21, to show the pigmented center and an outer layer of cholesterol. × 70.

FIG. 26. Carbonate spherolith from the interior of such a stone as is shown in Fig. 25. The calculus had been submitted to weak acid which dissolved out a carbonate matrix, causing it to fall apart into cholesterol plates and organic débris. Such treatment left only the stained shadow of the spherolith. Its concentric striation, plainly visible in the original, cannot be seen in the photograph. × 150.



(Rous, Drury, and McMaster: Causes of gall stone formation. II.)

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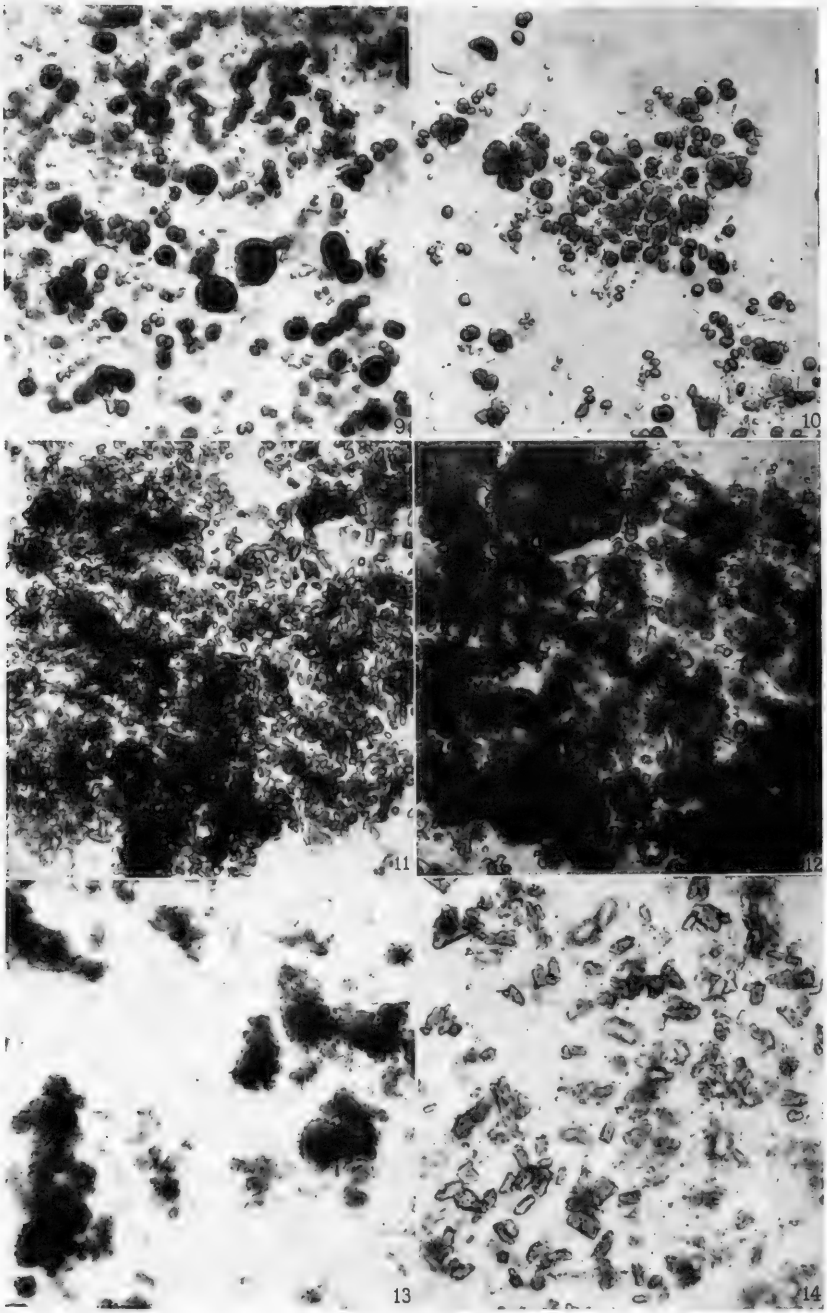

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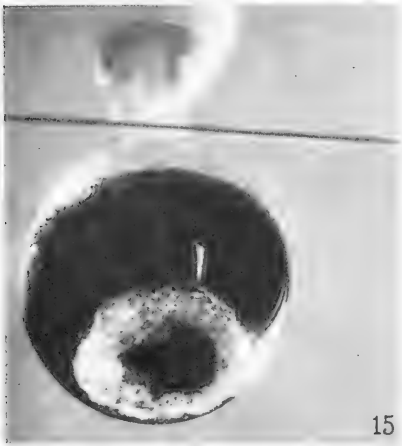

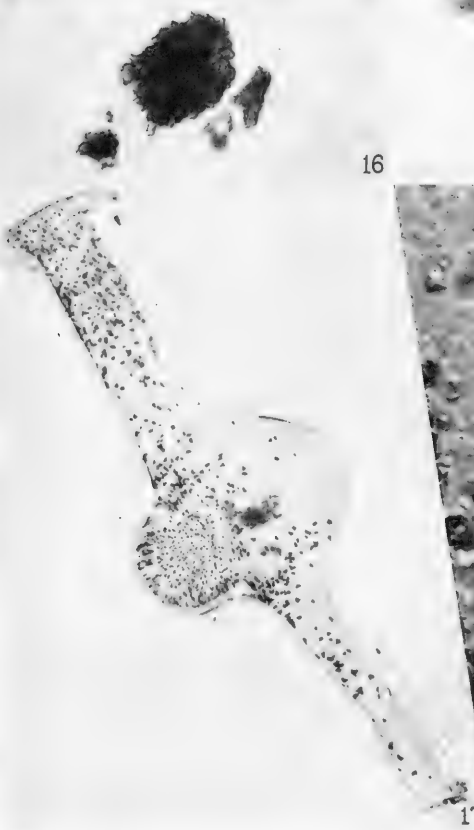

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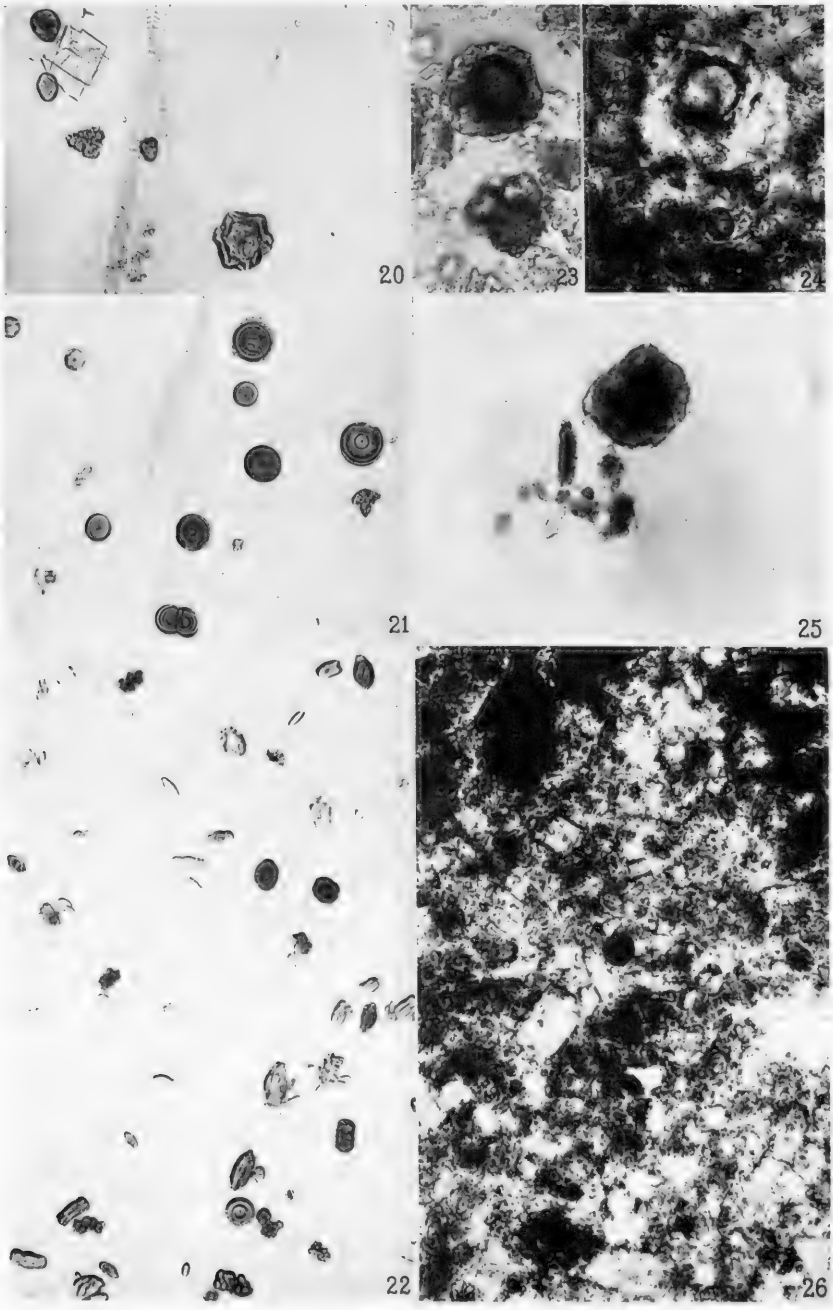

(Rous, Drury, and McMaster: Causes of gall stone formation. II.)

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THE RÔLE OF ANHYDREMIA AND THE NATURE OF THE TOXIN IN INTESTINAL OBSTRUCTION.

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The reader is referred to an article by Ellis[1] for the most recent comprehensive review of the literature on experimental intestinal obstruction. The following report includes work on the water loss, chemistry of the blood, and nature of the toxin produced in the obstructed small intestine of dogs.

Anhydremia.

That anhydremia plays a leading rôle in the causation of symptoms and that it may even be the cause of death in intestinal obstruction was maintained by Hartwell and Hoguet[2] and supported by the work of McLean and Andries[3] and by that of Bacon, Anslow, and Eppler.[4] Draper[5] found an average dehydration of 10 per cent, but this also occurred when the dogs were fasted or subjected to pilocarpine and then without symptoms comparable with those seen in intestinal obstruction. Therefore, he concludes that water loss plays an unimportant part in intestinal obstruction. Draper's work was confirmed by Dragstedt, Moorhead, and Burcky,[6] who observed fatal obstruction in dogs without much vomiting or other loss of fluid.

During the past 2 years we have produced obstruction of the small intestine in a large number of dogs.[7] Changes in water content of

* Research fellow of the National Research Council.

[1] Ellis, J. W., *Ann. Surg.*, 1922, lxxv, 429.

[2] Hartwell, J. A., and Hoguet, J. P., *J. Am. Med. Assn.*, 1912, lix, 82.

[3] McLean, A., and Andries, R. C., *J. Am. Med. Assn.*, 1912, lix, 1614.

[4] Bacon, D. K., Anslow, R. E., and Eppler, H. H., *Arch. Surg.*, 1921, iii, 641.

[5] Draper, J. W., *J. Am. Med. Assn.*, 1914, lxiii, 1079.

[6] Dragstedt, L. R., Moorhead, J. J., and Burcky, F. W., *J. Exp. Med.*, 1917, xxv, 421.

[7] All operations were performed under ether anesthesia.

TABLE I.

Water Content of Blood and Tissues before and after Intestinal Obstruction.

Dog No.	Blood. Systemic. Before.	Blood. Systemic. After.	Blood. Portal. Before.	Blood. Portal. After.	Spleen. Before.	Spleen. After.	Muscle. Before.	Muscle. After.	Liver. After.	Weight. Before.	Weight. After.	Vomiting.	Remarks.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*kg.*	*kg.*		
1	77.4	74.6	78.2	73.1	76.3	76.3	72.2	66.3	72.7			+	Inversion of both ends of duodenum. Length of life 1 day. No peritonitis. Killed.
2	74.9	72.2	75.0		74.8	75.6	71.5	68.7	71.5	14.25		++++	Inversion of both ends of duodenum. Length of life 1 day. No peritonitis. Killed.
3	77.4	74.8	77.5		77.8	77.0	63.3	63.3	71.2	16.0		++++	Inversion of both ends of duodenum. Length of life 1 day. No peritonitis. Killed.
4	77.4	76.0	77.1	75.5	77.8	76.4	72.5	70.2	73.3	19.0	17.75	++++	Inversion of both ends through upper jejunum. No peritonitis. Lived 1 day.
5	73.9	73.4	72.7	73.9	77.2	75.5	71.9	72.1	72.2	19.6	18.9	+++	Inversion of both ends of terminal ileum. Lived 2 days.
6	77.1	76.9	76.4	77.0	76.2	76.3	69.8	68.3		22.9	22.3	0	" " " " " lower " " 3 " No peritonitis. No vomiting.
7	74.0	73.8	74.1	73.3	76.4	76.3	71.2	72.4		18.0	17.2		Clamping of terminal ileum. Lived 2 days. No peritonitis.
8	75.5	75.1	75.0	75.0	76.1	75.3	72.4	73.1	71.6	17.6	16.7	++++	Wire around terminal ileum. Duration of life 3 days.
9	78.9	79.3	78.5		75.2	77.3	71.8	71.8	71.9	12.2			" " " " " " " 3 " No peritonitis.
10	77.1	77.1	77.5	76.6	76.7	76.8	73.8	73.6	72.5	16.3	15.7		Wire about terminal duodenum. Anterior gastrojejunostomy. Beginning diffuse peritonitis.

the blood and tissues, and changes in blood chemistry were determined in about thirty animals. The data of ten fairly representative experiments are presented in Table I. The specimens obtained after obstruction were removed shortly before the death of the animal.

The figures in Table I show that a moderate loss of water may occur, especially in high obstruction, but it is an inconstant phenomenon apparently not related to the severity of the symptoms. In fact we have observed most severe symptoms without any apparent desiccation whatsoever.

The water determinations were made by quickly removing blood or tissues, hashing the latter, weighing in stoppered bottles, and drying to constant weight over phosphorus pentoxide in a high vacuum at room temperature. This procedure usually required 3 days.

Chemistry of the Blood (Table II).

Urea and Non-Protein Nitrogen.—An increase occurred in all instances but with few exceptions the elevation was slight and apparently proportional to the severity of the symptoms. The rise in non-protein nitrogen was almost entirely due to the increase in urea.

The increased non-protein nitrogen in intestinal obstruction has been recorded by a number of investigators. Tileston and Comfort[8] observed it in man; G. H. Whipple and collaborators[9] and Bacon, Anslow, and Eppler[4] noted an increase in the non-protein nitrogen in the blood of dogs. McQuarrie and Whipple[10] maintained that the increase of non-protein nitrogen is partly due to disturbed renal function, while Rabinowitch[11] observed good phthalein elimination and therefore believes that increased protein destruction is the cause and he is supported by Louria[12] who also determined the phthalein elimination in human intestinal obstruction.

Chlorides.—A marked decrease of blood chlorides occurred in nearly all our instances and this appeared to be related to the frequency of vomiting.

[8] Tileston, W., and Comfort, C. W., *Arch. Int. Med.*, 1914, xiv, 620.

[9] Cooke, J. V., Rodenbaugh, F. H., and Whipple, G. H., *J. Exp. Med.*, 1916, xxiii, 717.

[10] McQuarrie, I., and Whipple, G. H., *J. Exp. Med.*, 1919, xxix, 397.

[11] Rabinowitch, M., *Canad. Med. Assn. J.*, 1921, xi, 163.

[12] Louria, H. W., *Arch. Int. Med.*, 1921, xxvii, 620.

The decrease in chlorides is comparable with that observed by Macallum and associates[13] and by Hastings, Murray, and Murray[14] in experimental pyloric obstruction. Haden and Orr[15] conclude from their experiments on canine and human intestinal obstruction that renal insufficiency may be excluded as causative of the high non-protein nitrogen in the blood and that the lowering of blood chlorides is not to be accounted for by the loss of hydrochloric acid through vomiting.

TABLE II.

Chemistry of the Blood before and after Intestinal Obstruction.

Dog No.	Amount per 100 cc. of blood.													
	Urea nitrogen.				Non-protein nitrogen.				Chlorides.		Fibrin.		Calcium.	
	Systemic.		Portal.		Systemic.		Portal.		Systemic.		Systemic.		Systemic.	
	Before	After.	Before.	After.	Before.	After.	Before.	After.	Before.	After.	Before.	After.	Before.	After.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg*
1	14.0	45.3				93.2		96.2	560	454				
2	14.0	21.0	15.0	21.8	34.1		34.4		546	385				
3	17.5	33.0	17.1		35.4		34.6		539	385				
4	15.4	19.7	16.2	23.9	33.1	43.3	35.0	48.3	596	552				
5	11.2	19.1	11.9	21.0	33.0	38.0	30.3	37.1	586	378				
6	13.5	16.8	14.8	18.5	34.2	44.4	35.0	44.4	506	506				
7	13.0		14.9		38.0	49.2	38.0	50.2	532	379				
8	19.6	42.0	18.6	44.3	37.6	59.7	39.3	66.3	541	419				
9	14.0	25.3	14.0		34.9	46.1	34.9	46.1	581	394				
10	9.3	115.6	9.3	116.2	30.0	156.2	28.6	156.2	512	369				
11		39.0				73.1					530	719	9.1	9.7
12		29.8				61.2					450	579	9.9	9.9
13		170.0				231.0						1,468		11.1

Other Blood Constituents.—Fibrin estimated in three instances was found to be increased. The increase in fibrin is hard to explain in view of the delayed coagulation time in experimental obstruction observed by Whipple, Stone, and Bernheim[16] and others. The calcium content of the serum was unchanged. Cholesterol and sugar were determined a few times but no significant change was noted.

[13] Macallum, W. G., Lintz, J., Vermilye, H. N., Leggett, T. H., and Boas, E., *Bull. Johns Hopkins Hosp.*, 1920, xxxi, 1.

[14] Hastings, A. B., Murray, C. D., and Murray, H. A., Jr., *J. Biol. Chem.*, 1921, xlvi, 223.

[15] Haden, R. L., and Orr, T. G., *J. Exp. Med.*, 1923, xxxvii, 365.

[16] Whipple, G. H., Stone, H. B., and Bernheim, B. M., *J. Exp. Med.*, 1913, xvii, 286.

The Toxin.

Roger and Garnier[17] obtained a toxic substance from the contents of the obstructed small intestine of rabbits by precipitation with alcohol and extraction of the precipitate with water. G. H. Whipple and assistants[18] proceeded in a similar manner but further purified the material by precipitation with ammonium sulfate and showed that the toxin was resistant to heat, soluble in water, insoluble in alcohol, undigested by proteolytic ferments, non-dialyzable, and precipitated by half saturation with ammonium sulfate. Owing to its resistance to the action of trypsin they thought that the toxin was a heteroproteose. Gerard[19] suggested that the toxin might be a peptamine containing histamine because when hydrolyzed with 20 per cent hydrochloric acid it retained its toxic properties and yielded a fraction which gave the diazo reaction.

The present work includes an investigation of the properties and analyses of the toxic material, attempts at fractionation, and a study of the individual fractions. Owing to the difficulty of obtaining the substance (about 1 gm. of purified material was obtained from the obstructed intestine of fourteen dogs) it has only been possible to carry out a limited number of experiments. The method employed was that described by Ellis[1] with additional steps to further purification.

The mucous membrane and content of the obstructed intestine were boiled in 200 to 300 cc. of water, then filtered through gauze and cotton, and treated with 5 volumes of 95 per cent alcohol. The precipitate dissolved in water was boiled with 1 gm. of magnesium sulfate, then filtered through paper, and reprecipitated with 5 volumes of alcohol. The material from fourteen dogs obtained in this manner was dissolved in a small amount of distilled water and dialyzed in a collodion sac against distilled water for 5 days. Thymol was used as a preservative. The neutral mixture, now free from magnesium sulfate, was centrifuged and the supernatant fluid strongly acidified with acetic acid boiled for a few minutes, cooled, and again centrifuged. The clear fluid was again precipitated with 5 volumes of alcohol. The next morning the solid was removed and dried over phosphorus pentoxide in vacuum.

The dry substance weighed 1 gm. and possessed the following properties. 71 mg. dissolved in water and injected intravenously killed

[17] Roger, H., and Garnier, M., *Rev. méd.*, 1906, xxvi, 953.

[18] Whipple, G. H., Rodenbaugh, F. H., and Kilgore, A. R., *J. Exp. Med.*, 1916, xxiii, 123.

[19] Gerard, R. W., *J. Biol. Chem.*, 1922, lii, 111.

a dog weighing $5\frac{1}{2}$ kilos in $2\frac{3}{4}$ hours. Death was preceded by retching, vomiting, wide respiratory excursions of the abdominal type, bloody diarrhea, and tenesmus. At autopsy there was hemorrhage into the mucous membrane of the entire small intestine but this was most marked and confluent in the duodenum. Punctate and non-confluent hemorrhages were also present in the colon. The toxic substance is soluble in water with slight opalescence which disappears on adding a trace of alkali. Precipitation results when a large excess of acetic acid is added. It gives a strong Molisch reaction, also Millon, diazo, weak biuret, and Hopkins-Cole tests.

Analysis.—The substance was dried in vacuum over sulfuric acid at 100°C. 0.1002 gm. gave 0.1708 gm. of carbon dioxide and 0.062 gm. of water. 0.0958 gm. required 8.25 cc. of 0.1 N acid when analyzed according to Kjeldahl's method. 0.0479 gm. was dissolved in 5 cc. of water. 2 cc. of this solution yielded 0.270 cc. of nitrogen gas at 763.4 mm. and 25° when analyzed according to Van Slyke's method for amino nitrogen.

Found. Carbon 46.48 per cent.
Hydrogen 6.92 " "
Nitrogen 12.05 " "
Amino nitrogen before hydrolysis 0.69 per cent.
" " after " 8.00 " "
Ash 7.58 per cent.

Hydrolysis.—0.554 gm. of dry substance was hydrolyzed by boiling with 20 per cent hydrochloric acid under reflux for 36 hours. The acid was removed by evaporation *in vacuo* and the residue was dissolved in 50 cc. of slightly alkaline water. 2 cc. of this solution were analyzed for amino nitrogen according to Van Slyke's method and gave 3.25 cc. of nitrogen gas at 760.4 mm. and 28°. Before hydrolysis 5.7 per cent of the nitrogen was in the amino form; after hydrolysis 66 per cent was present as amino nitrogen. The hydrolysate gave a strong reaction for phosphate with ammonium molybdate and nitric acid but no reaction for sulfates. 8 cc. of the solution were precipitated with phosphotungstic acid according to the method of Hanke and Koessler.[20] The small precipitate which formed on standing was freed from phosphotungstic acid with barium hydroxide; the filtrate

[20] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1920, xliii, 543.

was evaporated, alkalinized with sodium hydroxide, and extracted with amyl alcohol. The extract failed to give the diazo reaction for histamine.

Toxicity of the Dialysate.—The fluid obtained during the first 2 days of dialysis of the toxin was evaporated to a small volume and injected intravenously without toxic effects.

Toxicity of the Alcoholic Filtrate.—The alcoholic filtrate obtained after precipitation of the intestinal contents of three obstructed dogs was evaporated in vacuum, taken up in water, filtered, sterilized, and injected into the vein of a dog without toxic effect.

Toxicity of the Hydrolysate.—25 cc. of a solution of hydrolyzed toxin (equivalent to 0.277 gm. of dry substance) were injected into the vein of a 5 kilo dog without toxic effect.

Presence of Histamine in the Alcoholic Liquors.—The alcoholic filtrate obtained from precipitation of the contents of the intestine of twelve obstructed dogs was evaporated to 1,500 cc., acidified with dilute hydrochloric acid, filtered, and treated as follows:

Precipitation with basic lead acetate, filtration, and removal of the excess of lead with hydrogen sulfide, evaporation of the clear filtrate to 500 cc., and precipitation with phosphotungstic acid according to Hanke and Koessler's method. The phosphotungstate was decomposed with barium hydroxide, the barium phosphotungstate removed by filtration, and the filtrate freed from barium with sulfuric acid. The clear solution was evaporated to 400 cc. and precipitated with silver nitrate and barium hydroxide according to the Kossel and Kutscher process. At first sufficient barium hydroxide was added to produce a neutral reaction and the resulting precipitate was removed; then an excess of silver nitrate and barium hydroxide was added and the precipitate treated with sulfuric acid and hydrogen sulfide in the usual manner. The filtrate was evaporated to 10 cc., made alkaline with 3 gm. of sodium hydroxide, and extracted with amyl alcohol. The alkaline solution gave a strong diazo reaction, thus indicating the presence of considerable histidine. The alcoholic solution was extracted with 100 cc. of 0.1 N sulfuric acid. The acid liquid was neutralized with barium hydroxide and filtered. The clear barium-free filtrate was concentrated to 4.5 cc. 1.5 cc. of this solution injected intravenously into a guinea pig killed by asphyxia in 3 minutes. 0.5 cc. killed a large guinea pig by asphyxia in 5 minutes. Autopsy showed marked distension of the lungs.

The histamine content of the fluid was determined colorimetrically by von Fürth and Hryntschak's[21] method. It was found that each

[21] von Fürth, O., and Hryntschak, T., *Biochem. Z.*, 1914, lxiv, 172.

cubic centimeter contained 0.82 mg., or about 4 mg. in the total amount of fluid.

Test for the Presence of Toxin in the Liver of Obstructed Dogs.—A watery extract of the livers of two obstructed dogs was precipitated with 5 volumes of alcohol, the precipitate was dissolved in water and injected intravenously into a dog without producing signs or symptoms indicative of toxicity.

Test to Determine Whether the Pancreas Is Necessary for the Formation of Toxin.—Toxin prepared from the obstructed intestine of a depancreatized dog produced death when injected intravenously into another dog.

Fractionation Experiments.—2.5 gm. of purified material, prepared as already described from the obstructed intestine of nineteen dogs, were dissolved in 150 cc. of water. The turbid solution, which changed to a colloidal suspension on adding 17 cc. of glacial acetic acid, was dialyzed in collodion sacs against distilled water for 24 hours. The dialysate when evaporated to a small volume gave a positive test for phosphate but negative biuret, Fehling, Millon, and diazo reactions, and was not toxic when injected intravenously into a dog.

The material in the collodion sacs was now further dialyzed against three 4 liter portions of distilled water; it consisted of an upper clear layer, a middle layer containing suspended matter, and a thick sediment. The upper layer was filtered and precipitated with 5 volumes of 95 per cent alcohol (fraction 1). The remainder of the material in the dialyzing tubes was centrifuged, and the supernatant fluid filtered and precipitated with 5 volumes of alcohol (fraction 2). The sediment in the centrifuge tube was stirred with water and centrifuged for 2 hours without obtaining good sedimentation; the supernatant fluid was then poured off and precipitated with alcohol (fraction 3). The final residue in the centrifuge tube constitutes fraction 4.

Fraction 1 gave a slight biuret and Millon reaction and a strong Molisch and pentose reaction (Bial). On adding the diazo reagent no reaction was obtained at first but on long standing a brown color gradually developed. When evaporated with dilute nitric acid a yellow residue remained which became orange-red on adding sodium hydroxide and bluish purple when heated with this reagent, thus

indicating the presence of purines. Dried to constant weight in vacuum over phosphorus pentoxide at 100°, the substance analyzed as follows: 0.0496 gm. required 2.7 cc. of 0.1 N acid according to Kjeldahl's method. 0.0576 gm. was dissolved in 5 cc. of water; 2 cc. of this solution gave 0.25 cc. of nitrogen gas at 755 mm. and 21° when analyzed for amino nitrogen according to Van Slyke's method. Another 2 cc. portion of the solution was hydrolyzed by boiling with 25 per cent hydrochloric acid for 25 hours, neutralized, and diluted to 8 cc. 2 cc. of the dilution gave 0.53 cc. of nitrogen at 24° and 749 mm. of mercury.

0.0079 gm. of substance was dissolved in 3 cc. of 0.1 N sodium hydroxide. 1 cc. of this solution was mixed with 1 cc. of N sulfuric acid and hydrolyzed in a Folin sugar tube by immersion in boiling water for 4 hours.[22] The solution was neutralized and its reducing power determined according to the method of Folin and Wu.[23]

Found. Nitrogen 7.63 per cent.
Amino nitrogen before hydrolysis 0.61 per cent.
" " after " 5.05 " "
Reducing power expressed as glucose 28.8 per cent.

0.100 gm. of this fraction injected intravenously killed a dog weighing 6.4 kilos in 3½ hours. Autopsy showed characteristic hemorrhages in the mucosa of the small intestine.

Fraction 2 gave all the reactions obtained with the first fraction and analyzed as follows: 0.0647 gm. analyzed according to Kjeldahl's method required 5.04 cc. of 0.1 N acid. 0.0057 gm. was dissolved in 3 cc. of 0.1 N sodium hydroxide. 1 cc. of this solution was hydrolyzed by heating with 1 cc. of N sulfuric acid and the reducing power determined according to Folin and Wu.

Found. Nitrogen 10.91 per cent.
Reducing power after 3 hours hydrolysis 18.4 per cent.
" " " 4 " " 18.7 " "

0.110 gm. was heated for 4 hours with 10 cc. of 0.5 N sulfuric acid with reflux condensation, then neutralized and injected into a dog

[22] Previous experiments with yeast nucleic acid had shown that this period of hydrolysis yielded the maximum amount of reducing substance.

[23] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

weighing 3.1 kilos without any toxic manifestations. 0.100 gm. heated for 4 hours as above but with 10 cc. of 0.5 N sodium hydroxide was neutralized and injected intravenously into a dog weighing 2.8 kilos without any toxic symptoms.

Fraction 3 gave tests identical with those obtained with fraction 1, except that the biuret test was stronger. When dissolved in water it formed a turbid suspension which cleared on the addition of alkali but became cloudy again when acidified. This fraction, as well as all the others, was soluble in an excess of mineral acid. Dried in vacuum at 100° over phosphorus pentoxide it analyzed as follows: 0.0462 gm. gave 0.0046 gm. of $Mg_2P_2O_7$ after fusion with sodium hydroxide and sodium nitrate. 0.0931 gm. gave 0.1598 gm. of CO_2, 0.0506 gm. of H_2O, and 0.0061 gm. of ash when burnt in the combustion tube. 0.1127 gm. required 11.91 cc. of 0.1 N acid when analyzed according to Kjeldahl's method. 0.0606 gm. was dissolved in 5 cc. of water containing a trace of alkali. 2 cc. of this solution (0.02424 gm. of substance) gave 0.28 cc. of nitrogen at 20° and 762 mm. when analyzed according to the method of Van Slyke for amino nitrogen. Another 2 cc. portion (0.02424 gm.) was hydrolyzed with boiling 20 per cent hydrochloric acid for 62 hours, then neutralized, and made up to 8 cc. 2 cc. of this solution (0.00606 gm.) gave 0.87 cc. of nitrogen at 22° and 750 mm. 0.010 gm. was dissolved in 4 cc. of 0.1 N sodium hydroxide. 1 cc. of this solution (0.0025 gm.) was heated for 4 hours with 1 cc. of N sulfuric acid and its reducing power determined according to the Folin and Wu method.

Found.	Carbon	46.80 per cent.
	Hydrogen	6.08 " "
	Nitrogen	14.79 " "
	Phosphorus	2.77 " "
	Ash	6.55 " "
	" calculated from phosphorus content 6.34 per cent.	
	Amino nitrogen before hydrolysis 0.658 per cent.	
	" " after " 7.98 " "	
	Reducing power expressed as glucose 11.2 per cent.	

0.025 gm. was injected intravenously into a small dog weighing 3.6 kilos and produced toxic effects including vomiting and bloody diarrheal stools. The dog recovered after 5 days.

Fraction 4 was washed with alcohol and dried. It contained 12.04 per cent of nitrogen and gave the same tests as the previous fractions. It was not further investigated.

DISCUSSION.

The data regarding the toxin appear to support most of the conclusions of G. H. Whipple and collaborators; but the greater solubility of the substance in alkali and strong mineral acids than in weak acids, its non-coagulability by heat, its chemical reactions and analyses including the presence of phosphoric acid and pentose characterize it as a nucleoprotein. The toxic material does not contain histamine free or combined as suggested by Gerard. Though a small amount of histamine is present in the content of the obstructed bowel it appears insufficient to contribute to its toxic nature.

The more soluble fractions of the toxin contain less nitrogen and more carbohydrate relatively speaking and may therefore be cleavage products derived from the original material by hydrolytic removal of the purines or pyrimidines, thus leaving substances richer in pentose and poorer in nitrogen.

SUMMARY.

1. Data are presented which show that anhydremia does not play a leading rôle in the causation of symptoms in intestinal obstruction.

2. Chemical changes in the blood corresponding with those noted by previous investigators were observed.

3. Additional data regarding the chemistry of the toxin found in the obstructed intestine of dogs were obtained.

MICROBIC VIRULENCE AND HOST SUSCEPTIBILITY IN PARATYPHOID-ENTERITIDIS INFECTION OF WHITE MICE.

III. The Immunity of a Surviving Population.

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Topley[1] and Amoss[2] have both observed that recurrent outbreaks of mouse typhoid in an experimentally controlled community flourish more readily and are much more fatal among the fresh recruits than among the older surviving individuals left over from previous epidemics. The experiments described in this paper relate to the nature of the resistance displayed by this latter group.

The past histories of the surviving individuals of epidemics generally considered are uncertain. It is assumed that taking all the survivors together, certain of them have entirely resisted infection, certain have been infected and have recovered, others carry the disease in a more chronic form, while still others have escaped exposure entirely. In planning the present experiments, exposure to infection was insured by the administration of the cultures *per os*, and chronic forms of infection were detected by adequate bacteriological methods. In this way the number of survivors was reduced to mice which had (*a*) actually resisted or (*b*) recovered from the infection.

The purpose of the first experiment was to determine the protecting effect of different strains of paratyphoid-enteritidis bacilli against a subsequent dose of the epidemic mouse typhoid culture, M. T. II.

[1] Topley, W. W. C., *J. Hyg.*, 1921, xx, 103. Topley, W. W. C., and Wilson, G. S., *J. Hyg.*, 1923, xxi, 243.

[2] Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 45.

Immunity among Survivors to Bacilli.

Experiment 1.—According to the purpose of this experiment, each of six series of mice of similar age and weight was given by stomach tube one of six paratyphoid-enteritidis strains in a known dose which varied from 3,000,000 to 6,000,000 bacteria. The technique of this procedure has been discussed, together with the characteristics of the various strains employed: Mouse Typhoid I (M. T. I), Mouse Typhoid II (M. T. II), *B. enteritidis* (Gaertner), *B. aertrycke* (mutton), *B. pestis caviæ*, and *B. paratyphosus* B.[3,4]

60 days after this injection, about 10 per cent of the *B. enteritidis* series, 20 per cent of the M. T. I series, 30 per cent of the M. T. II and of the *B. aertrycke* series, and 70 per cent of the *B. paratyphosus* B and of the *B. pestis caviæ* series were alive. These mice were temporarily considered to be survivors. Each series was placed two per jar and with twenty normal control mice were given *per os*

TABLE I.

Summary of Protocols of Surviving Mice Inoculated with Strain M. T. II.

Preliminary bacterial strain.	No. of mice.	Mice developing positive blood cultures.	Mice dead at 8 wks.	Mice surviving at 8 wks.
		per cent	*per cent*	*per cent*
No preliminary cultures (controls).....	20	75	80	20
B. pestis caviæ.....	27	33	52	48
" *paratyphosus* B.....	32	31	52	48
M. T. I.....	15	13	33	67
" II.....	32	6	31	69
B. aertrycke (mutton).....	22	4	27	73

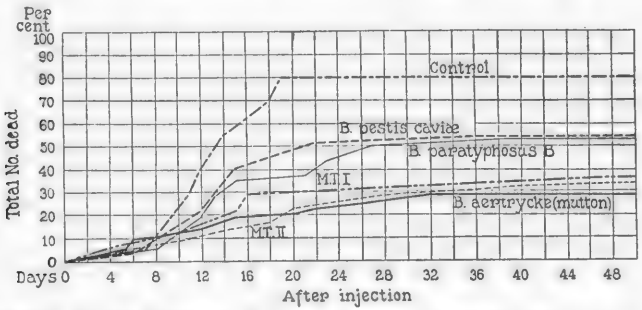
a fixed dose, 4,000,000 bacteria, of the epidemic mouse typhoid strain, M. T. II. Blood cultures were taken at frequent intervals, and all fatal cases were autopsied and cultured. Whenever the original bacillary strain administered for immunizing purposes was recovered, the mice yielding it were considered to be chronic cases and not true survivors. All such mice were excluded from the final computations.

Table I and Text-fig. 1, in which the duration of life among the survivors is compared with that of the controls, are taken from the last of three tests, all alike in respect to the treatment given, the number of mice used, and the end-results. Examination of the protocols and chart shows unmistakably that mice which have passed

[3] Webster, L. T., *J. Exp. Med.*, 1923, xxxviii, 33.
[4] Webster, L. T., *J. Exp. Med.*, 1923, xxxviii, 45.

through and survived an otherwise infecting dose of paratyphoid-enteritidis bacilli given *per os* exhibit greater resistance to a second dose of a related bacillus than do normal mice not previously exposed to infection.

In addition, a second effect is discernible; namely, that the amount of resistance displayed by each group of survivors is more directly related to the virulence of the strain of bacillus originally administered than to an antigenic similarity of this first strain to the second strain employed. Thus *Bacillus paratyphosus* B, which is of low virulence, yielded at first relatively a large group of survivors but at the end of the experiment relatively a small number of ultimate



TEXT-FIG. 1. The resistance of groups of mouse typhoid survivors to a second dose of similar organisms.

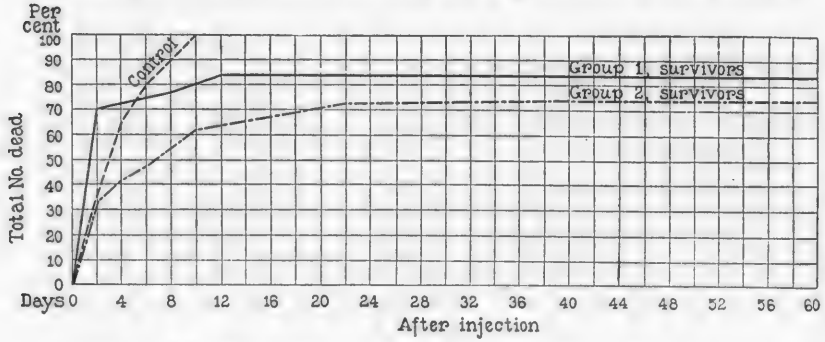
survivors to the second inoculation with Strain M. T. II; and likewise the survivors of *Bacillus pestis caviæ* which not only is also of low virulence but is antigenically identical with Strain M. T. II. On the other hand, the mice surviving the more virulent *Bacillus aertrycke* (mutton) and Strain M. T. II, itself, proved far more resistant to the second administration made with M. T. II. In a like manner, Strain M. T. I, which is highly virulent but antigenically dissimilar from Strain M. T. II, left survivors which resisted, in the way of the preceding examples, the second administration of Strain M. T. II. Hence it appears that the virulent strains of bacilli originally inoculated eliminated the susceptible individuals from the respective series of mice with the result that the survivors consisted of the naturally resistant part of the populations on which

the bacillary Strain M. T. II exerted little effect. The fact should be mentioned that too few individuals survived the original *Bacillus enieritidis* inoculation to provide survivors for the second M. T. II test.

Parallel observations by Pritchett have enabled us to eliminate the possibility of cross-protection phenomena in this experiment.[5]

Resistance of Survivors to Chemicals.

The inherent qualities on which resistance to infection is based may consist, therefore, not only of specific but also of non-specific factors. We have previously pointed out[6] that any large number of mice divides



TEXT-FIG. 2. The resistance of mouse typhoid survivors to mercury bichloride.

itself automatically into three groups according as they (*a*) become infected and die, (*b*) become infected and recover, and (*c*) escape infection altogether when given a fixed dose of mouse typhoid bacilli. A somewhat similar grouping was determined to take place when a toxic chemical such as mercury bichloride was substituted for living bacteria.[4] The question now arose as to the manner in which the surviving mice, in the sense of Experiment 1, would react to the administration of this toxic drug.

Experiment 2.—The total number of mice tested in this experiment was 81, divided as follows: 13 surviving one dose of mouse typhoid bacilli (Group 1); 48 surviving two doses (Group 2); and 20 normal or control mice of similar weight (Group 3). Each mouse received 0.0016 gm. of mercury bichloride in 0.5 cc. volume of distilled water *per os* by stomach tube.

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[5] Pritchett, I. W., *J. Exp. Med.*, 1924, xxxix (in press).

[6] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 231.

Text-fig. 2 clearly shows that the mice which survived one or more doses of mouse typhoid bacilli resist a toxic dose of mercury bichloride better than do the normal controls. This result supports and extends the results of our previous experiments with this chemical and indicates anew that in escaping or recovering from infection, certain non-specific factors operate just as they operate in preventing a fatal drug intoxication.

"Resistant" versus "Recovered" Survivors in Relation to Infection.

The data presented above emphasize the influence of a factor which for want of a better name we have called non-specific in the determination of infection or intoxication and recovery from those conditions. Still other evidence bearing on the same point will now be presented. It is to be regretted that the material available was not more voluminous, but in spite of its limited size, it has seemed to yield a significant fact.

Experiment 3.—From a series of earlier experiments, eleven mice were chosen which had survived an infecting dose of Strain M. T. II *per os* by stomach tube. Stool cultures, blood cultures, and agglutinin tests had been frequently performed. Eight of these mice had passed the bacilli with the feces for a few days but had shown no positive blood cultures (twelve tests) and no homologous agglutinins (four tests) during the 8 weeks period. Three of these mice, on the other hand, had shown symptoms of disease and had recovered. They had passed bacilli for 4 to 6 weeks; blood cultures, positive for 4 weeks, had become negative and had remained so for 4 weeks. The serum of each had agglutinated the homologous Strain M. T. II in a dilution of 1:5,000 or 1:10,000.

These eleven mice, together with eleven controls of the same weight, were injected intraperitoneally with 1 cc. of an 18 hour broth culture of Strain M. T. II diluted 1:500.

The controls were all dead on the 6th day; no deaths had occurred among either group of survivors during a 30 day period of observation.

The results of this experiment indicate that not only are the "recovered" survivors which show specific agglutinins more resistant to Strain M. T. II injected intraperitoneally than are normal control mice, but also that the "resistant" survivors which have failed to respond either with evidences of infection or the production of agglutinins are equally refractory.

Response of the Chronically Infected Mice.

In addition to the "survivors" among the inoculated mice, which term as used in this paper applies to those which resisted as well as those which recovered from infection, there is a third group of mice which we designate as "chronic cases." These animals, few in number, which apparently had recovered, were reinoculated *per os* 8 weeks after the original inoculation. They succumbed not only to the second but to the first inoculation or to the combined action of the two infections. The determination in each instance depended upon the cultures recovered at autopsy. Apparently these animals

TABLE II.

Chronic Cases among Several Survivor Series.

Series No.	Survivor group.	Total No. of survivors.	No. of chronic cases.
1	M. T. I	20	3
2	" I	20	1
2	Enteritidis.	8	2
3	M. T. I	15	1

were able to cope with one infectious process but not with two. The original infection was indeed not mastered in part without damage, because the organs (liver and spleen) showed chronic lesions; hence they readily succumbed to a second inoculation which either revived the original infection or started a second equally fatal one (Table II).

CONCLUSIONS.

Mice which survive a preliminary dose *per os* of paratyphoid-enteritidis bacilli are more resistant to a second dose *per os* of a similar epidemic mouse typhoid strain than are mice which have received no preliminary culture.

The amount of this resistance is related to the pathogenicity of the preliminary strain more than to its antigenic similarity to the second strain.

Mice which survive a preliminary dose *per os* of paratyphoid-enteritidis bacilli are more resistant to a lethal dose of mercury bichloride *per os* than are mice which have not received the preliminary bacterial culture.

Mice which have resisted and mice which have recovered from a preliminary dose *per os* of paratyphoid-enteritidis bacilli are more resistant to a lethal intraperitoneal dose of an epidemic mouse typhoid strain than are mice which have not received the preliminary culture.

Mice in which the preliminary dose of paratyphoid-enteritidis bacilli has induced a chronic infection readily succumb to a second dose of such bacilli.

These findings indicate that the resistance mechanism of the host contains important non-specific factors which vary in degree with the individual mice.

STUDIES ON VIRULENCE.

II. The Increase in Virulence in Vitro of a Strain of Pneumococcus.

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This paper is the second of a series on the study of virulence of microorganisms grown *in vitro*. Paper I[1] is a preliminary investigation and has to do with the influence of virulence on microorganisms transferred during the logarithmic increase phase—the period of optimum multiplication activity. In recent years, considerable attention has been directed toward the activity of so called young microorganisms as compared to the old culture. Chesney[2] has shown that the 6 to 8 hour growth of pneumococci has greater infectivity than the culture of 18 to 24 hours. Chick,[3] who worked with *Bacillus paratyphosus*, alleges that young microorganisms are more resistant to antiseptics than old ones, while Sherman and Albus,[4] in a publication on the physiological youth of bacteria, conclude that differences exist between young and mature bacterial cells, the newly formed cells passing through a period of physiological youth. They base their views upon the varied actions of mature and young bacteria exposed to 2 per cent sodium chloride, phenol, and low temperatures, the young cell being more susceptible to these agents injurious to life. The lag in rate of growth following inoculation—an inability of organisms to continue cell division at a constant rate after being introduced into a change of environment—can be explained from the standpoint of age of the cells, for Barber[5] and McKendrick and Pai[6] have proved

[1] Felton, L. D., *Bull. Johns Hopkins Hosp.*, 1923, xxxiv, 262.

[2] Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.

[3] Chick, H., *J. Hyg.*, 1908, viii, 92.

[4] Sherman, J. M., and Albus, W. R., *J. Bact.*, 1923, viii, 127.

[5] Barber, M. A., *J. Infect. Dis.*, 1908, v, 379.

[6] McKendrick, A. G., and Pai, M. K., *Proc. Roy. Soc. Edinburgh*, 1910–11, xxxi, 649.

that bacterial lag is absent when young cultures are used for inoculation. Young organisms may be thought then to possess greater reproductive activity than old organisms, or, perhaps, to become more easily adapted to environmental conditions inimical to the multiplication of older cells. If we may assume that a fundamental difference exists in bacteria at varying intervals of time after cell division—variation in age and function—analogous to those found in the stages of animal life (infancy, childhood, maturity, old age) a possibility is foreseen that microorganisms transferred in the vigor of their youth may adapt themselves more easily to conditions which favor an increase or decrease in virulence; that is, the microorganism, when young, may perhaps be more readily immunized, according to Welch's hypothesis, against the substances or conditions unfavorable to existence, or, to express the matter from another standpoint, be trained to live on such a food supply as will fit it for life on living animal tissue. In a previous study, it was observed that the youth of the cell is a factor in the rate of reaction to environment. This fact prompted the undertaking of a study to determine what changes, if any, in the virulence of bacterial cells might be found when transfers are made during the period of greatest growth activity; namely, youth.

Estimation of Virulence.

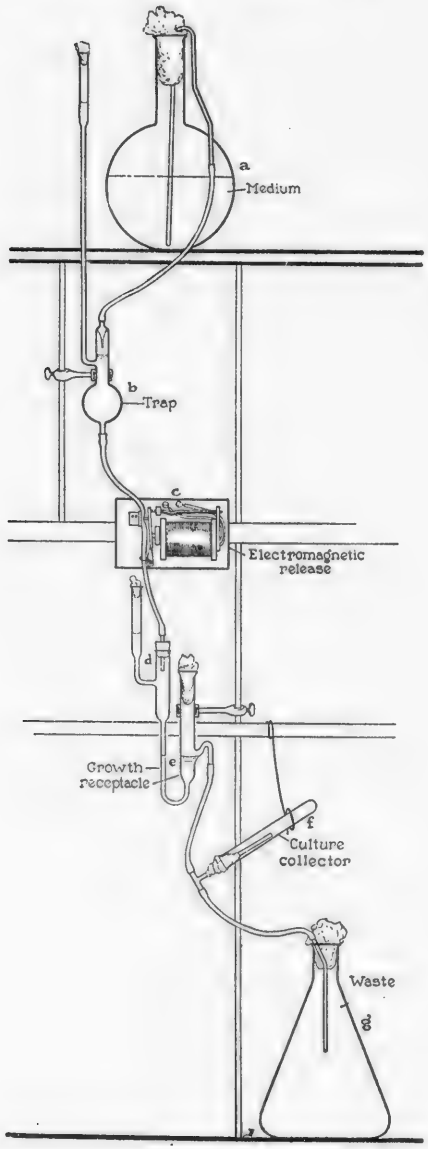
The virulence of the strains of pneumococci employed in the present work was estimated in terms of the minimum number of organisms which caused the death of a mouse in from 36 to 72 hours. The actual titration of virulence was done by injecting mice intraperitoneally (three or four for each test) with varying numbers of organisms, the successive dilutions of which represented a logarithmic series, as for example, 1:10, 1:100, 1:1,000, 1:10,000. The minimum lethal dose was assumed to be the least number of organisms that caused death consecutively in the series of mice injected. This procedure was used to minimize probable error. The irregularities in the mortality of the experimental animals that were injected with doses of organisms which differed so widely as those of a logarithmic series may be ascribed perhaps more to variations in the individual susceptibility of the animal than to irregularities in the virulence of the pneumococci. For virulence of a culture at a given time is a constant factor when dealing with the experimental infection; the variable is the animal. Whence it follows that an estimation of virulence necessitates taking into consideration the sequence and the regularity of death in the series of mice injected with graded doses of organisms. The number of organisms that were injected for the estimation of virulence of any culture was calculated by the plate method, through the use of serum agar plates (pH = 7.4) with three different dilutions.

Apparatus.

Two changes were made in the apparatus as described in the former study. Instead of a dropping device to regulate the flow of medium, an electromagnet clamp controlled by a time-clock was employed. In the growth receptacle previously used, the straight arm overflow did not permit of transfers at short intervals when media were studied in which the pneumococci grew with difficulty, because the number of organisms which remained in the growth receptacle was insufficient to reinoculate the fresh medium. For this reason, a short siphon was made to take the place of the straight arm overflow, and thus a mixing of fresh culture was assured before the overflow began. It was then found possible to make flushings of the device as often as every half hour and still maintain growth of the cocci. A typical arrangement of the apparatus is shown in Text-fig. 1.

The large siphon flask (*a*) at the top contains the medium which enters by rubber tubing into a trap (*b*) through a capillary tube of smaller bore than the outlet. This trap, used for the purpose of insuring a constant pressure head, is in turn connected by means of rubber tubing to a capillary tube (*d*) of known bore, which is held in the inlet arm of the growth receptacle by means of a rubber stopper. The spring on the electromagnet (*c*) closes the rubber tubing between the trap and the growth receptacle (*e*). From the small siphon on the growth receptacle, a rubber tubing leads to the waste flask (*g*) through the culture collector (*f*), this being a T with a long arm, around the upper part of which cotton is wound as a plug for a test-tube of usual size. In operation, the electromagnet is connected with a timing device so that at fixed intervals the spring is released and a quantity of the medium, which will depend upon the size of the capillary and length of time of release, flows into the receptacle. The fluid rises in this vessel until the siphon is started and then overflows into the waste. Organisms are obtained when desired from the growth receptacle by placing the collector so that it points downward and clamping the tubing between it and the waste; the test-tube of culture is taken off and another, after being well flamed, is put in its place. Sterilization of the apparatus is accomplished by autoclaving, either in units or as a whole. Little difficulty has been experienced with contamination. Any trouble met with was caused either by defective cotton plugs or by rubber tubing containing a spore-bearing bacillus.

The growth receptacle made from 15 mm. tubing has a capacity up to the siphon of 7 cc., to overflow of 12 cc., inlet and outlet tubes being 3 mm. in diameter. The siphon is 10 mm. high. The capillary inlet in the rubber tubing varies, of course, with the kind of medium and the length of time of release of the electromagnet spring. With the present apparatus, the 1 mm. tubing allowed 15 cc. of plain broth to pass through in 1 second, or about 14 cc. of sterilized skimmed milk.



TEXT-FIG. 1. Apparatus designed for study of virulence. A typical set-up.

Choice of Medium.

Whatever the conception of virulence, most investigators conceive a virulent organism to be one that will grow within the animal body and be capable of producing a characteristic lesion or death of the host. This may be true whether virulence is considered a state of affairs brought about by overcoming conditions found in the living host detrimental to bacterial growth, Welch's hypothesis, or as a state developed through the influence of specific food such as that contained in animal tissue. If we recall the demonstrated fact that animal passage as a rule increases virulence, and assume further that the growth of the microorganism on animal tissue is responsible for such a biological variation, it follows logically that a medium for the study of virulence *in vitro* must be made from animal tissues. Since milk contains the food elements needed for maintenance and growth in the animal body and is in addition one of the most readily available forms of animal secretions having the constituents necessary for life, it was selected as medium for this study. Unless otherwise stated, the milk was freshly pasteurized skimmed milk, delivered packed in ice from a commercial pasteurizing depot. With one exception, milk from a single batch was employed for each experiment. Sterilization was usually accomplished in an Arnold sterilizer for 55 minutes on 3 successive days. This length of time was found necessary for effective sterilization, for the reason that 30 to 35 minutes were required to heat a large flask of medium to 100°C.

Experiment 1. Effect of Interval of Transfer on Increase of Virulence.

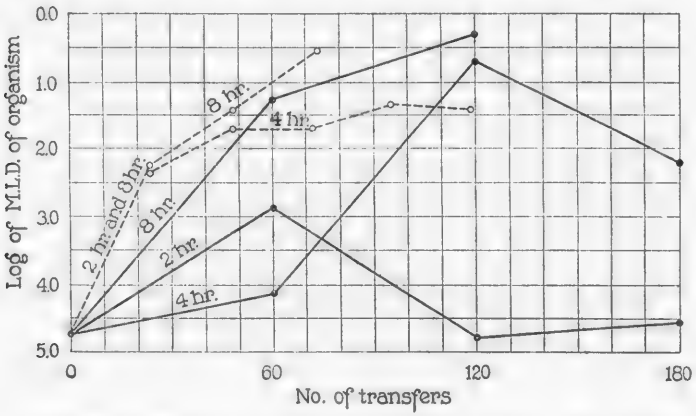
The purpose of the following experiment was to establish the optimum point of the growth curve, as determined by interval of transfer, for enhancing virulence.

Two experiments (Text-fig. 2), involving the same culture grown on two different lots of skimmed milk, were carried out with transfers made at 2, 4, and 8 hour intervals. The strain of pneumococcus used was the Neufeld, Type I, which had been made avirulent by 24 hour transfers on horse serum (Stryker;[7] Wadsworth and Kirkbride[8]). The culture grew characteristically turbid on plain broth, was bile-soluble, and true to type.

[7] Stryker, L. A., *J. Exp. Med.*, 1916, xxiv, 49.

[8] Wadsworth, A. B., and Kirkbride, M. B., *J. Exp. Med.*, 1918, xxviii, 791.

The results as represented on the chart reveal the possibility of markedly increasing the virulence for mice of a strain of pneumococcus by growth *in vitro*, the degree of enhancement depending to some extent on the interval between transfers. In the study represented by the solid lines, the 8 hour interval is optimum and the 4 hour almost as favorable, while the 2 hour interval acts to bring about some increase of virulence with a subsequent lessening. The experiment illustrated by the dotted lines demonstrates somewhat different results; the 4 hour interval is as favorable in this case as the 8 hour interval, but the 2 hour approaches the efficacy of the 4 hour interval

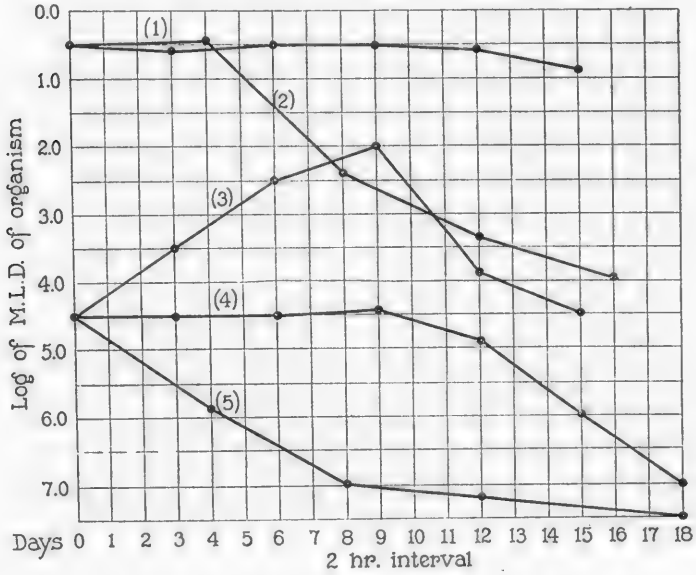


TEXT-FIG. 2. Effect of variations in interval of transfer; milk medium.

of the previous experiment. The lack of correspondence in these two experiments is rather perplexing and indicates that some factor other than the interval of transfer is operative in the increase in virulence of pneumococci in milk. The average number of organisms per cubic centimeter was approximately the same in both experiments, 4×10^5, the number in the culture of a 2 hour period being somewhat less. Among possible reasons for the irregularity in the enhancement of virulence shown in these experiments, that of variability in the character of the milk suggested itself. To ascertain whether different lots of this medium vary in suitability, Experiment 2 was carried out.

Experiment 2. Effect of Different Lots of Milk.

Four different lots of milk of the same H ion concentration (pH = 6) were studied in their influence on a highly virulent and a relatively avirulent strain of pneumococcus. The organisms used were the avirulent Neufeld, Type I, of Experiment 1 (Text-fig. 3, Nos. 3, 4, and 5), and the same strain with initial virulence maintained by occasional animal passage (Text-fig. 3, Nos. 1 and 2).



TEXT-FIG. 3. Influence upon virulence of different lots of milk. The numbers 1 and 3 represent the experiments made with a single sample of milk; the numbers 2, 4, and 5 represent those on three different lots.

The results depicted upon Text-fig. 3 signify that milk does not supply a uniform medium for the increase of virulence. The virulence of a strain of microorganism which was highly virulent to begin with was maintained or was decreased, while that of an avirulent strain was either increased, maintained, or decreased when it was grown on the various samples of milk. The question naturally arises as to what cause the varied results may be attributed to. Pasteurized skimmed milk has undergone certain changes caused by heat and

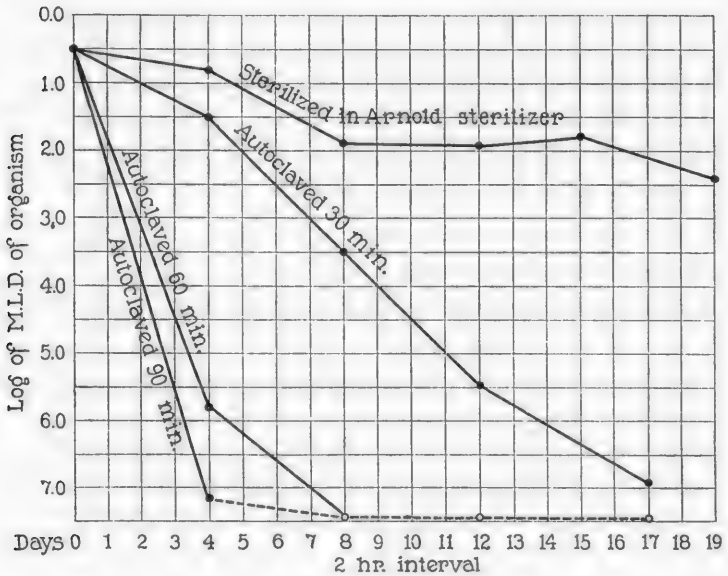
during the process of cream separation. Whole milk is difficult to handle with the apparatus used in this study, so before making an investigation of this question, the effect of varying degrees of heat on the suitability of skimmed milk for maintaining and increasing the virulence of pneumococci was studied.

Experiment 3. Effect of Heating Milk on the Ability to Influence Virulence in Vitro.

Two strains of pneumococci from one culture (Neufeld) were used, a virulent strain (Text-figs. 7 and 4) and a strain that remained avirulent in spite of all attempts to alter its virulence by either *in vivo* or *in vitro* methods. The same batch of milk was divided into four lots and each portion was sterilized by heat for varying lengths of time; one lot (Text-figs. 7 and 4) was sterilized in an Arnold sterilizer on each of 3 days for 55 minutes; one autoclaved 30 minutes at 17 pounds; one autoclaved 60 minutes at 17 pounds; and the fourth portion autoclaved 90 minutes at 17 pounds pressure. All were adjusted to pH = 7.4 after heating.

The rate of growth of the pneumococci was about the same in the four experiments, variation in numbers in the milk sterilized in the Arnold sterilizer and in milk autoclaved for 90 minutes averaging about 20 per cent, with the higher count in the former. The avirulent culture remained avirulent throughout the experiment; 1 cc. direct from the growth receptacle or from an 18 hour culture did not produce a fatal infection. The effect of varying degrees of heat influenced the suitability of the milk for maintenance of virulence. Although this particular sample of milk heated in an Arnold sterilizer did not supply conditions appropriate in a 2 hour interval of transfer for the maintenance of the original virulence of this strain of pneumococci, it is clear that the suitability of the medium for preserving virulence was decreased in proportion to the length of time it was autoclaved. Though growth occurred in all four lots of milk of this experiment, it may be concluded that milk subjected to high temperatures is so altered in its constitution that virulent pneumococci grow on it when transferred at 2 hour intervals lose their virulence for mice. The number of organisms in each portion of milk, regardless of the method of sterilization or length of time of exposure to heat, was approximately the same; the difference in the cultures was the rate at which changes in virulence took place. Not improbably, a heat-labile substance in

milk which is essential for maintaining or increasing virulence was destroyed. The avirulent strain of pneumococci remained avirulent throughout these experiments, as might be expected.



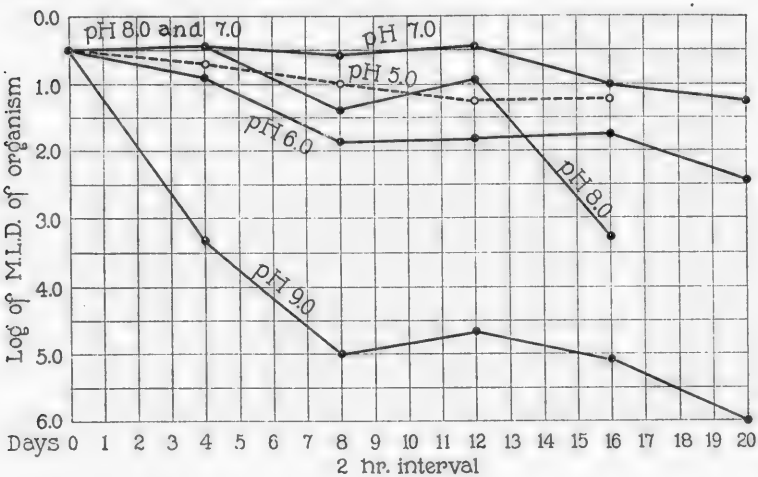
TEXT-FIG. 4. Effect of heat upon the suitability of milk to increase virulence.

Experiment 4. Effect of Hydrogen Ion Concentration.

The same strains of virulent and of avirulent organisms were used in this experiment as in the preceding one. Adjustment of the milk to various H ion concentrations was carried out after sterilization. Duplicate flasks of five different H ion concentrations, pH = 5, 6, 7, 8, and 9, were prepared from a single batch of milk. No attempt was made to estimate the pH nearer than 0.2. In a study of the effect on virulence of H ion concentration of plain broth, to be reported later, it was found that growth of pneumococci occurred at an H ion concentration as low as pH = 5. Inasmuch, however, as Avery and Cullen[9] have shown that the optimum H ion concentration for the initiation of the growth of pneumococci in plain broth is pH = 7.8, a large number of organisms, 5 cc. of a young broth culture, was used to assure inoculation of media of such wide variation

[9] Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1919, xxx, 359.

in H ion concentration as those between pH = 5 and pH = 9. Growth began immediately in them, although some variation in the number of organisms occurred in the milk of the various H ion concentrations. The flask of pH = 5 averaged 4×10^6, pH = 6 averaged 4.5×10^6, pH = 7 averaged 5.8×10^6, pH = 8 averaged 2.5×10^6, and pH = 9 averaged 2.2×10^6. The optimum H ion concentration for multiplication was thus found in the medium of pH = 7, the pH = 6 producing a little less growth. While the media titrated to pH = 5, 8, and 9 all supported good growth, there was a gradual falling off in numbers in the last two (pH = 8 and 9) after the 8th day. On the average, the final H ion concentrations of the cultures in the growth receptacles were as follows: pH = 5 remained 5.0; pH = 6 dropped to 5.8; pH = 7 dropped to 6.4; pH = 8 dropped to 7.2; pH = 9 dropped to 7.8.



TEXT-FIG. 5. Effect of the H ion concentration of milk on virulence.

The results in regard to virulence (Text-fig. 5) are definite and surprising. Virulence was maintained at a practically constant level in milk varying as widely in H ion concentration as pH=5, 6, and 7 for 192 transfers; at pH=8 in the same number of transfers a tenfold decrease in virulence occurred; while with the pH=9 flask, the decrease in virulence began immediately and continued throughout 20 to 30 times to be less than that of the organisms of the other flasks of milk. Obviously, under the conditions of this experiment, the H ion concentration of milk between pH=5 and 7 exerted little

influence detrimental to virulence of pneumococci. On the other hand, milk of pH = 8 and 9 caused a definite decrease in the virulence of this organism. The fact that no increase in virulence was obtained with the avirulent culture implies that this culture, which was granular, bile-soluble, and typical as far as could be determined, was in such a state that no development of virulence was possible by the method employed.

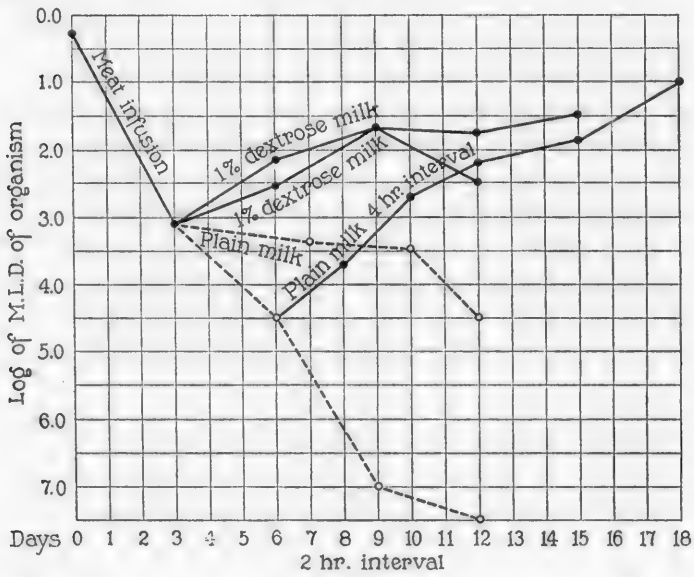
Experiment 5. Pneumococci Attenuated in Meat Infusion Again Become Virulent on Milk.

In another study[10] it has been shown that virulent pneumococci grown on meat infusion or plain broth suffer a rapid decrease in virulence so that 1×10^7 or 1×10^8 organisms were not fatal for mice in this state of attenuation. All efforts to restore the lost virulence of the culture failed. To determine just when a virulent pneumococcus grown on meat infusion loses its virulence to a degree at which it can no longer be restored, an experiment with meat infusion was planned involving the inoculation of this medium with a virulent organism and transfers from time to time to milk in an attempt to restore the lessened virulence before it had been entirely lost.

A culture was taken from the meat infusion at a time when 1,000 organisms were required to cause the death of a mouse and transferred to three automatic machines, two of which contained 1 per cent dextrose milk and one plain milk, all with the same interval of transfer as the meat infusion (2 hour). The plain milk (Text-fig. 6) arrested the decrease in virulence for a time, while the dextrose milk produced a condition favorable for increase. Again, when the virulence of the organisms grown on meat infusion had decreased so that not less than 50,000 organisms proved fatal for a mouse, the culture was inoculated into plain milk medium with 4 hour intervals of transfer. In 2 days of transfers, a definite increase of virulence was noted which continued without a break for 12 days, when the experiment was terminated. No other trials to increase virulence were made until the culture grown on meat infusion had become so avirulent that 10,000,000 organisms failed to produce a noticeable effect on a mouse. When in this state of attenuation, growth of the organism on dextrose milk with periods of transfer of 2, 4, 8, and 12 hours failed to increase virulence.

[10] Felton, L. D., *J. Exp. Med.*, 1924, xxxix, 155.

In association with change in virulence, the strain of organism grown in meat infusion had developed at the end of the experiment peculiar characteristics; in its avirulent state the capsule was absent, it sedimented during growth like a hemolytic streptococcus, and grew i n long chains. It retained, however, the ability to produce methemoglobin with rabbit blood corpuscles and was bile-soluble. Its behavior toward immune sera is noteworthy (Table I). Whereas the usua agglutination titer for the original strain of Neufeld pneumococcus



TEXT-FIG. 6. Restoration of lost virulence by growth on milk.

with the corresponding immune sera was 1:64 with no agglutination occurring in Type II or Type III sera, it was now flocculated by a 1:32,000 dilution of Type I serum and by dilutions of 1:4,000 and 1:1,600, respectively, of Type II and Type III sera. Apparently the specificity is retained in high dilution, but the organism is much more sensitive to agglutination. The work of De Kruif[11] describing

[11] De Kruif, P. H., *J. Exp. Med.*, 1922, xxxvi, 309.

two types of organism in a culture of bacillus of rabbit septicemia, virulent and avirulent, with varying morphological characteristics, having a different isoelectric point, led us to perform an experiment similar to his with this avirulent organism. The difference in the behavior between the avirulent pneumococcus and the original culture was similar to that of the two types of organism described by De Kruif. The Neufeld virulent strain grew evenly turbid, while the avirulent one settled to the bottom of the culture tube as did, respectively, Types D and G of De Kruif. In a single experiment with the phosphate buffer series, the isoelectric point of the virulent pneumococcus culture and that of the same culture when rendered avirulent differed significantly. Other strains

TABLE I.

Changes in the Behavior of a Pneumococcus with Immune Sera on Becoming Avirulent in Meat Infusion Media.

Types of sera.	Dilution of immune sera.						
	1:500	1:1,000	1:2,000	1:4,000	1:8,000	1:16,000	1:32,000
I	+++	+++	+++	+++	+++	+++	+++
II	+++	+++	+++	++			
III	+++	+++	++				

Dilutions were made by halving the concentration from 1:2 in series to 1:32,000; agglutination occurred in all types of sera alike up to 1:500.

of organisms in various stages of virulence have behaved in a like fashion; details of this work will be published elsewhere.

Experiment 6. Increase of Virulence by a Pure Line Avirulent Pneumococcus.

The results of the preceding experiment raise the question as to the mechanism involved in the increase of virulence on growth *in vitro* or *in vivo*. Is the process one of adaptation of the bacterial cell to the condition supplied by animal life, or is virulence a more or less fixed characteristic of certain cells which enables them to live in or on living animal tissue, the avirulent organisms being destroyed while the virulent ones survive, with cell division furnishing a new genera-

tion, the offspring of which possesses both weak and strong cells? It is not our intention to do more than inquire into the mechanism of increase of virulence by the method described in this paper.

To obtain a pure line strain of pneumococcus, the Barber pipette, used so successfully by the originator to pick single microorganisms,[12] was resorted to. A strain of Neufeld pneumococcus, rendered so nearly avirulent by transferring it after the ordinary fashion in horse serum broth medium that 1×10^3 cocci

TABLE II.

Virulence of Pure Line Strains of Pneumococci Picked from One Mother Strain.

Culture.	Duration of life.							
	Dilution of a 16 hr. culture.							
	0	1×10^{-1}	1×10^{-2}	1×10^{-3}	1×10^{-4}	1×10^{-5}	1×10^{-6}	1×10^{-7}
	hrs.	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*
N-1	S.*	S.	S.	S.	S.	S.	S.	S.
			days					
N-2	36	48	7	"	"	96	"	"
			hrs.					
N-3	36	48	48	48	48	S.	"	"
			days					
N-4	36	S.	6	S.	S.	"	"	"
			hrs.					
N-5	S.	"	48	"	"	"	"	"
N-6	24	36	48	48	48	48	48	60
N-7	36	48	48	S.	S.	S.	S.	S.
N-8	S.	S.	S.	72	"	"	"	"
N-9	"	"	"	S.	"	"	"	"
N-10	36	48	48	48	48	48	48	48
N-11	S.	S.	S.	S.	S.	S.	S.	S.

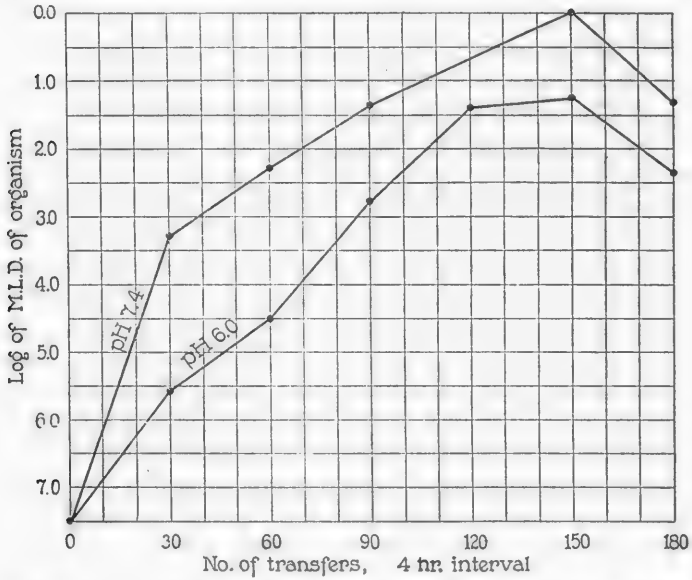
S. indicates survived.

were needed to kill a mouse, was used as the source from which to pick; picking was done from a 1:100,000 dilution of an 18 hour broth culture of the organism. Eleven takes (Table II) out of seventeen were obtained. The virulence for mice was determined for each pure line strain in a manner shown in Table II. Eight mice were used for each strain, the number of organisms injected into each animal representing a logarithmic series of dilutions beginning with an undiluted 18 hour culture.

[12] Barber, M. A., *Philippine J. Sc.*, 1914, ix, 307.

The outstanding fact from this part of the experiment is the demonstration of the existence of cells of varying virulence in the same culture. The variation is great, ranging from that of a strain of which 1 cc. of a 16 hour culture failed to cause the death of a mouse, to one of such a degree of virulence that 10 to 100 organisms were sufficient to cause the death of an animal.

Culture N-1 was chosen as a suitable, avirulent pure line strain for the purpose at hand. Although H ion concentration of milk influenced but slightly the virulence of a strain of pneumococci, it was thought advisable to use this medium unadjusted (pH = 6) and also adjusted to pH = 7.4.



TEXT-FIG. 7. Increase of virulence by a pure line avirulent strain in milk medium.

The results of these experiments are shown in Text-fig. 7. The virulence of the avirulent pure line strain progressively increased in milk adjusted to a pH = 7.4 until it reached its maximum, one diplococcus as determined by plating producing a fatal infection of a mouse. With the unadjusted milk, the rise in virulence was perhaps not so marked. The difference in the rate of increase in virulence in the two experiments is not sufficiently great to permit of any conclusion as to

the optimum H ion concentration of milk necessary to enhance virulence. The significant fact is that a pure line avirulent strain produced from a single diplococcus underwent a maximum increase in virulence when grown *in vitro*. To our knowledge, such an enhancement of virulence by *in vitro* methods has never been obtained prior to this study. The results indicate that the selection of fit cells is not the only mechanism operative in the increase or maintenance of virulence, and that perhaps adaptation is just as important as selection for change in this bacterial function.

DISCUSSION.

The method used does not permit of a comparison between the results thus far obtained and those reported by other investigators who made use of the usual bacteriological technique. In our work, warm medium is supplied to a large number of bacteria during a period with a nearly constant multiplication rate, in contrast to the ordinary transfer procedure of introducing relatively few organisms of varying growth activity into a new supply of medium, with the sudden environmental change entailed thereby. The number of pneumococci which remain after flushing the growth receptacle with the amount of medium equal to twice its capacity is about one-third of the number present just before flushing, a fact established both by counting the organisms before and after flushing of the growth receptacle and by introducing a dye, the color intensity of which could be readily estimated. It can thus be seen that the present method of study is to be thought of as representing a feeding procedure at different hours rather than one involving transfers at various intervals. How far the factor of large inoculum of young organisms and of residual medium after flushing the growth receptacle have influenced the results can only be speculated on until work now in progress is finished.

The impossibility of general deductions is apparent for the reason that the work was limited to one strain of organism owing to the length of time required to obtain significant results in each experiment. In subsequent work, however, other strains of pneumococci of different serological types have been found to act similarly to the one reported in this study, some cultures increasing in virulence maximally and others not at all. We believe that the negative results

obtained with certain strains of avirulent organisms do not imply a fixed avirulent state, but are indicative that the method of study does not furnish proper environmental conditions to restore lost virulence. It should also be borne in mind that a single species of animal host has been used. Although it is probable that facts established concerning the virulence for mice of pneumococci may be applied to other animal species, such a view involves the problem of animal specificity and pathogenicity, a field of investigation, which, however tempting and important, is foreign to the present study.

Whatever future work may develop, it has been established by the present study that the virulence of a single avirulent pure line strain of pneumococcus has been enhanced maximally by growth outside the animal body. This holds also for the mother culture which had become avirulent under the conditions of ordinary laboratory technique. The fact that individual diplococci, when propagated separately in an 18 hour culture were found to possess highly varying degrees of virulence raises the question as to the mechanism of the increase of virulence of pneumococci *in vitro* or *in vivo*; of whether the process is one of adaptation, or selection, or both. The development of virulence in a pure line avirulent pneumococcus *in vitro* implies an adaptative phenomenon, whether or not cognizance is taken of the rate of development of virulence or the finished virulent state. In the experiment represented in Text-fig. 7, it is seen that the virulence of a pure line avirulent strain developed slowly, which suggests a gradual alteration in the bacterial cell. However, if it is assumed that hereditary influence plays a rôle in the character of bacteria, it is possible to explain this experiment as either an adaptative or a selective process, the existence of the bacterial cell being the important issue, its environment, viewed from the standpoint of selection, determining whether the selected organism be virulent or avirulent, or, from the standpoint of adaptation, whether or not the organism be so altered that it becomes antibiotic or saprophytic in nature. A study of the exact mechanism of the change in virulence is, perhaps, not so important as investigations of causes of this change.

SUMMARY AND CONCLUSIONS.

1. An automatic apparatus is described by which a new supply of food can be furnished actively growing organisms at any desired interval of time—an automatic transferring device.

2. A single strain of pneumococcus, Type I, Neufeld, which had become avirulent for mice, acquired virulence of maximal degree when grown in the described apparatus with skimmed milk sterilized in an Arnold sterilizer as the medium. Transfers were made at intervals of 2, 4, and 8 hours. It is difficult to determine the best interval of transfer, but the 8 hour interval apparently is most suitable, with the 4 and 2 hour following in preference in the order named.

3. Pasteurized skimmed milk from a single dairy, but obtained on different days, when used as medium at a 2 hour interval, varied in effect on the virulence of pneumococci, the results showing that the virulence might be either increased, decreased, or maintained.

4. Skimmed milk heated for varying lengths of time, 30 minutes at 17 pounds pressure, or 60 or 90 minutes at the same pressure, lost its suitability for maintaining virulence of a pneumococcus when transferred every 2 hours, the effect being in direct proportion to the length of time the milk was heated.

5. The H ion concentration of milk had slight effect on virulence. A virulent strain of pneumococcus was grown in milk adjusted to pH=5, 6, 7, 8, and 9, transfers being made every 2 hours. Virulence was maintained to a like degree on milk titrated to pH=5, 6, and 7, but when the organism was grown in milk of pH=8 and 9, virulence decreased, more rapidly at pH=9 than at pH=8.

6. A pure line practically avirulent strain of pneumococcus picked by the Barber method, when grown in milk at a 4 hour interval of transfer, increased in virulence 10 million fold; that is to say, until one diplococcus would kill a mouse. Prior to this study, no record has been found in which the virulence of any microorganism has been increased to such a degree by an *in vitro* method.

STUDIES ON VIRULENCE.

III. Influence of Hydrogen Ion Concentration and of Ingredients of Plain Broth on the Virulence of Pneumococci.

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(Received for publication, August 9, 1923.)

In the preceding paper[1] it has been reported that the virulence of a single strain of pneumococcus was increased several thousand fold when grown on milk in an automatic transferring device by which young cells were assured for inoculation. The degree of increase depended upon the specimen of milk, the frequency of transfer, and the state of virulence of the organisms. It was further shown that pneumococci grown upon ordinary meat extract broth at a 2 hour interval of transfer rapidly lost their virulence for mice. According to many investigators microorganisms in general lose their virulence more or less gradually on cultivation by the ordinary laboratory technique. Eyre and Washburn (1897),[2] Cotoni (1912),[3] and Wadsworth and Kirkbride (1918)[4] made a special study of pneumococci. They demonstrated a decrease in virulence of this organism when grown on plain broth. Stryker[5] also has reported the loss of this biological property as well as an alteration in agglutinability when pneumococci are grown on specific immune sera. Increase of virulence of pneumococci, though but to a slight degree, by cultivation *in vitro*, has been reported by Cotoni and also by Wadsworth and

[1] Felton, L. D., and Dougherty, K. M., *J. Exp. Med.*, 1924, xxxix, 137.

[2] Eyre, J. W., and Washburn, J. W., *J. Path. and Bact.*, 1897, iv, 394; *Lancet*, 1899, i, 19.

[3] Cotoni, L., Le virulence des pneumocoques, Thèse de Paris, 1912, No. 78.

[4] Wadsworth, A. B., and Kirkbride, M. B., *J. Exp. Med.*, 1918, xxviii, 791.

[5] Stryker, L. A., *J. Exp. Med.*, 1916, xxiv, 49.

Kirkbride. It may be assumed, therefore, from the present studies and those of others that the fact is established that pneumococci grown *in vitro* will either decrease or increase in virulence according to the condition provided. It is the purpose of the present study to report an analysis of the influence on virulence of pneumococci of transferring at varying intervals in plain broth of various H ion concentrations and of the individual ingredients of plain broth. This analysis was undertaken as a step toward the recognition of conditions and chemical substances which influence the virulence of pneumococci.

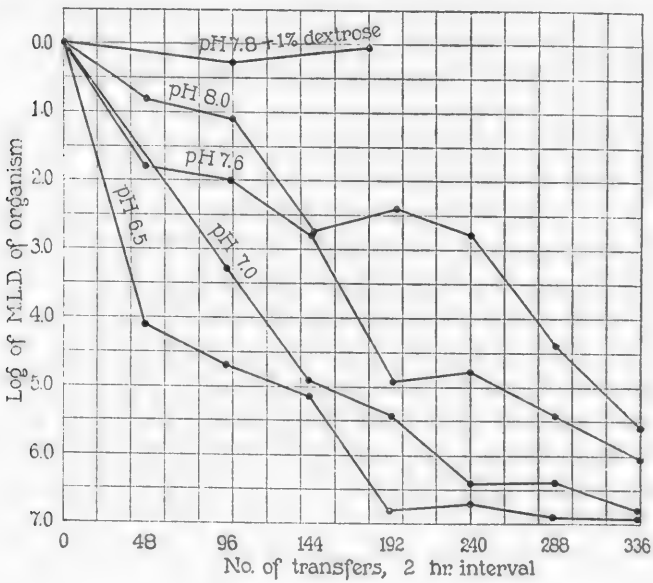
Experiment 1. Effect of H Ion Concentration on the Rate of Decrease in Virulence of Pneumococci Transferred at 2 Hour Intervals.

The apparatus and technique for this and the other experiments to be reported have been described in Paper II.[1] The strain of pneumococcus used in all experiments was the Neufeld, Type I.

The medium consisted of the usual meat infusion, 1 pound of lean beef to a liter, to which is added 1 per cent peptone and 0.5 per cent sodium chloride. The pH was adjusted so as to be 0.2 higher than the H ion concentration ultimately desired, and the medium so titrated was sterilized by free steam for 1 hour on 3 successive days. Generally the drop in H ion concentration during the process of sterilization compensated for the excess to which the medium had been adjusted; but when this did not occur, a readjustment was made with precautions to prevent contamination. Plain broth of four different H ion concentrations was studied, pH = 8, 7.6, 7, and 6.5. In order to assure growth, the different media were seeded with a large inoculum (2 cc.) of a young culture. Throughout this experiment, the average number of pneumococci per cc. amounted to, respectively, 25 million for pH = 6.6, 55 million for pH = 7, 50 million for pH = 7.6, and 35 million for pH = 7.8. The number of organisms, as well as the H ion concentration, remained practically the same throughout the experiment.

As can be seen from Text-fig. 1, the trend of virulence is downward, regardless of the H ion concentration of the medium, a confirmation of the general experience that growth of a microorganism in plain broth results in a decrease in virulence. These observations differ from those obtained by Wadsworth and Kirkbride who demonstrated clearly that a virulent organism can maintain its virulence for mice

when grown on plain broth or blood broth with an 8 hour interval of transfer. In a subsequent experiment this point will be taken up. The effect of H ion concentration on the virulence of this strain of pneumococcus is one of rate at which decrease occurs. It varies in direct ratio to the H ion concentration; the higher the concentration, the more rapid the attenuation. With milk this was not found to be the case, as was proven in Paper II, for the growth of the same



TEXT-FIG. 1. Effect of variation in pH; plain broth.

strain of pneumococci on lots of skimmed milk adjusted to H ion concentrations of pH = 5, 6, 7, 8, and 9 resulted in a decrease in virulence only in the milk of H ion concentrations of pH = 8 and 9, with practically no decrease at pH = 5, 6, and 7. The lack of correspondence, the reversed influence, even, in the effect of H ion concentration of plain broth and skimmed milk implies that other constituents of the medium may affect the virulence of pneumococci to a greater degree than the concentration of H ions.

Experiment 2. Effect of Constantly Changing H Ion Concentration of the Media.

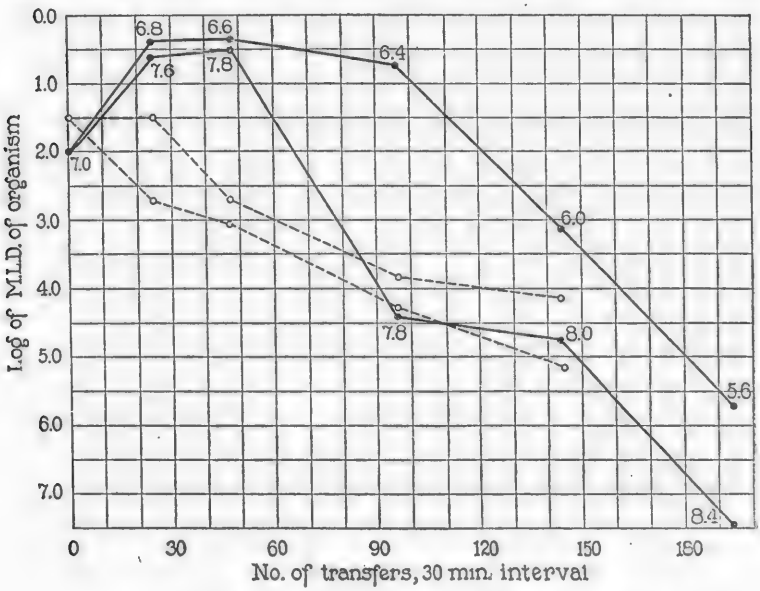
In the preceding experiment conclusions could not be drawn as to the relative influence of H ion and OH ion because of insufficient range in concentration of these ions. This fact, together with observations made during the study on milk, in which decrease in rate of virulence varied inversely with the H ion concentration, a result the opposite of that with plain broth, led us to devise an experiment in which the H ion concentration was gradually changed during the experiment, to either the acid or the alkaline side.

The procedure was as follows: medium of the siphon flask which emptied into the growth receptacle was replenished through a siphon connection to another flask containing medium of different H ion concentration, thus insuring a more or less gradual change in the pH of the medium supplied to the organism of the growth receptacle. Two experiments, the one involving a change to the acid and the other to the alkaline side of neutrality, were conducted, media of pH = 5 and pH = 9, respectively, being siphoned into media of pH = 7. The rate of change in H ion concentration of the medium and also the effect on virulence of such conditions is shown in Text-fig. 2. A 30 minute interval of transfer was used because it was thought that this short interval of transfer would render more evident the detection of the influence of the H ion or the OH ion. The Neufeld, Type I, strain which had lost some of its virulence by the ordinary stock method of transfer once a week on 10 per cent normal horse blood broth (24 hour incubation with subsequent storage 6 days in the ice box) was the one chosen for this experiment.

The results of the experiment (Text-fig. 2) are interesting from the standpoint of both virulence and agglutinability. The first 48 transfers, regardless of changes in the H ion concentration between pH = 6.6 and 7.8, revealed a noticeable increase in virulence with a final decrease in both series of tests. Here, as in the experiment reported on the influence of H ion concentration in milk, the OH ion is more detrimental to virulence than the H ion. The organisms in the growth receptacle were not counted as routine because growth was so slight that it was necessary to make inoculations from the machines into rabbit blood broth in order to have a sufficient number of organisms to estimate virulence. At the end of the experiment, however, counts were made of the number of organisms in the

respective growth receptacles, and there were found in the alkaline media 4,500,000 per cc. and in acid media 2,400,000 per cc.

How far the process of adaptation permitted growth in media of such wide variation in H ion concentration was not determined, nor was the alteration of the organisms, with the single exception of their reaction toward immune sera. This was taken up for the reason that in a 24 hour culture, the pneumococcus became auto-agglutinable as do hemolytic streptococci, an 8 hour culture, however,

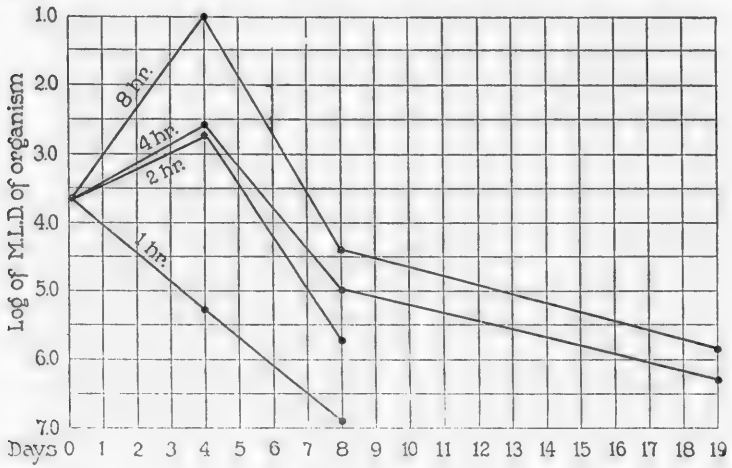


TEXT-FIG. 2. Effect of constantly changing pH in plain broth.

still remaining evenly turbid. The dotted lines on Text-fig. 2 represent logarithmically the titer of agglutination in Type I serum as determined by tests from time to time. The organism in the beginning of the experiment agglutinated at 1:50. The alkaline medium produced an immediate change in sensitivity toward agglutinins, which continued gradually until agglutination occurred in a dilution of immune sera of 1:200,000. The change in acid media was not so sudden and the organisms did not become so agglutinable, clumping in an approximate dilution of 1:20,000.

Experiment 3. Effect of Different Intervals of Transfer on the Virulence of a Somewhat Virulent Culture.

This experiment was prompted by the results of the preceding experiment, showing an initial rise of virulence followed by a decrease, regardless of H ion concentration, as further by the success of Wadsworth and Kirkbride in maintaining the virulence of pneumococci by transfer at an 8 hour interval, and by our own desire to determine in what way the age of the culture as judged by interval of transfer might influence virulence. The same strain of pneumococci used in the



TEXT-FIG. 3. Effect of varying the interval of transfer; plain broth pH 7.4.

previous experiment, but now in still less virulent condition, was employed. The intervals of transfer were four; namely, 1, 2, 4, and 8 hours. From Text-fig. 3 it can be seen that the virulence was affected differently when transfers were made at various intervals, 1 to 8 hours. The organism transferred every hour underwent a steady decrease in virulence in this medium, while at 2, 4, and 8 hour intervals of transfer, an initial increase in virulence occurred, followed by a decrease. These comparisons are somewhat misleading, inasmuch as tests for virulence were made with reference to days and not to number of transfers. Although no experiment

has been carried out to establish the fact, it has been observed in other studies that the initial rise in virulence on plain broth seemingly is dependent upon the number of transfers and independent of intervals of transfer from 1 to 8 hours. For example, in the experiment just described, in which a 30 minute interval of transfer was employed, a rise of virulence was noted after 48 transfers. It is possible that had the organism in the 1 hour machine been tested for virulence after 48 transfers, an initial increase of virulence might have been found. Although there was an initial increase in virulence with intervals of transfer of 2, 4, and 8 hours, the fact that virulence of the pneumococci eventually decreased irrespective of the interval of transfer makes it difficult to reconcile the present work with that of Wadsworth and Kirkbride who found it possible to maintain the virulence of several strains of pneumococci by transfers every 8 hours either on plain broth or rabbit blood broth. Inasmuch as our results have been amply confirmed and those obtained by Wadsworth and Kirkbride are equally definite, the disparity can, no doubt, be assigned to differences in technique.

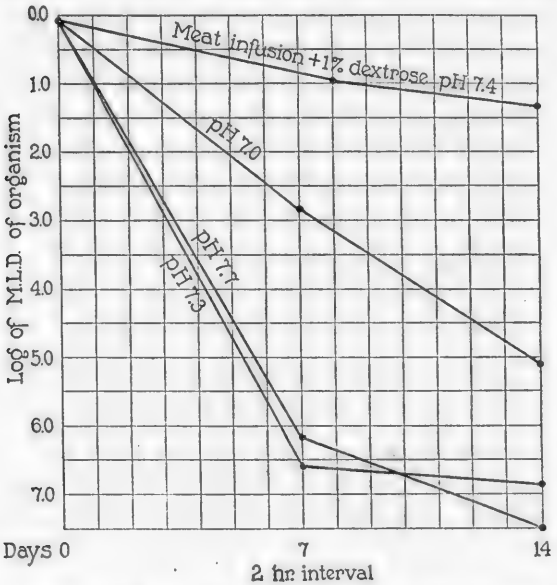
Experiment 4. Effect of Meat Infusion on the Virulence of Pneumococci Transferred at 2 Hour Intervals.

It is apparent at this point in our study that plain broth of a wide range of H ion concentration is not a suitable medium for maintenance of virulence, even when transfers are made while the organism is young. This is true despite the initial rise in virulence observed in the experiment. The unsuitability of meat infusion to maintain, much less increase permanently the virulence of the pneumococcus led us to undertake an analysis of the ingredients of plain broth to determine effects of meat infusion or of peptone on virulence.

The meat infusion used in this experiment consisted of 1 pound of lean beef to a liter, infused in the ice box overnight, with the addition of 0.5 per cent sodium chloride and 0.2 per cent disodium phosphate. Portions of this medium were adjusted to various H ion concentrations, pH = 7, 7.3, and 7.7. The Neufeld, Type I, pneumococcus, made virulent by animal passage, was employed. Owing to the press of other studies, estimation of virulence at frequent intervals

was impossible; however, it was found that the virulence of the pneumococcus was decreased (Text-fig. 4), the rate of decrease here again, as in milk, following in inverse ratio to the H ion concentration.

Meat infusion under the conditions of the experiment does not supply the proper food elements or environmental condition for the maintenance of virulence of pneumococci; but growth was abundant, so it may be assumed either that the meat infusion is lacking in some



TEXT-FIG. 4. Effect of variation in pH; meat infusion.

substance or condition which is necessary for pneumococci to retain their virulence, or that substances or conditions are present which rob them of their antibiotic faculty. An experiment was carried out to test whether or not a carbohydrate, dextrose, which is split by pneumococci, would provide the means to maintain virulence. This was done because of work carried out by one of us on metabolized media[6] in which it was shown that media exhausted by growth of

[6] Felton, L. D., *Bull. Johns Hopkins Hosp.*, 1923, xxxiv, 313.

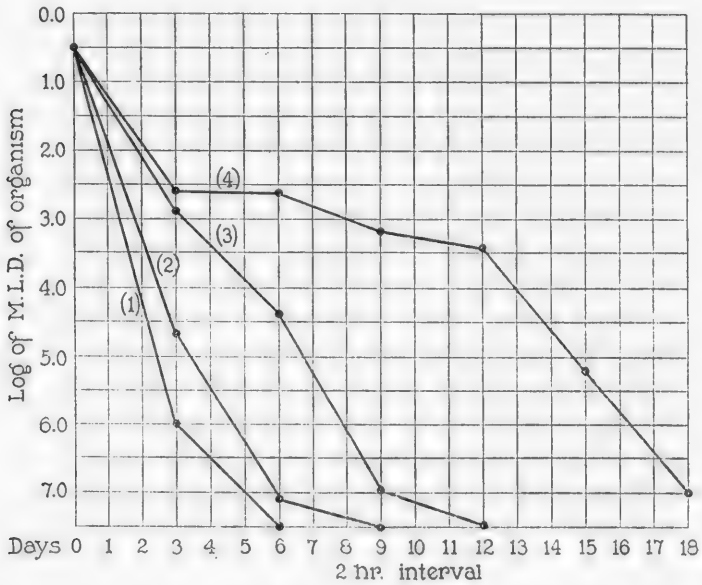
an organism could be revived, so to speak, by the addition of a carbohydrate normally utilized by the microorganism. Accordingly, dextrose was added to meat infusion and also to plain broth to make a 1 per cent solution, and these media placed in the usual automatic transfer apparatus were inoculated with highly virulent pneumococci. The results indicate (Text-figs. 1 and 4) that dextrose, added both to plain broth and meat infusion, so alters the condition of these media that the virulence of pneumococci grown thereon is maintained for a considerable length of time. It is possible that lack of carbohydrate in meat infusion will account for the failure to maintain virulence of pneumococci. On the other hand, since a maximal growth occurred in broth and meat infusion without dextrose, it seems possible that some chemical substance antagonistic to the virulence of the organism may be present in the media.

Experiment 5. Effect of Concentration of Meat Infusion on the Virulence of Organisms Transferred at 2 Hour Intervals.

The assumption that meat infusion in the usual concentration (1 pound to a liter) either lacks a substance or condition necessary for maintenance of virulence, or that there exists in it something detrimental to the virulence of this organism is open to experimental test. Dextrose added to plain broth or meat infusion, as recorded in the previous experiment, does away, at least partially, with the effect of these media to diminish virulence. If it be true that the infusion lacks some substance necessary to sustain virulence, meat infusion of a high concentration ought not to be any more destructive to this form of bacterial activity than is ordinary infusion, and perhaps not so destructive. On the other hand, should a substance antagonistic to virulence be present in the meat infusion, one would expect that the higher the concentration the more pronounced the action. With this question in mind, the following experiment was planned and carried out (Text-fig. 5).

Four lots of beef infusion of different concentrations were prepared: 3 pounds to a liter, 2 pounds to a liter, 1 pound to a liter, and $\frac{1}{2}$ pound to a liter, all adjusted to pH = 7.4. The number of organisms growing in these media varied from time to time. No. 1 ($\frac{1}{2}$ pound to a liter) averaged 30 million, No. 2 (1 pound to a liter) averaged 40

million, No. 3 (2 pounds to a liter) averaged 44 million, and No. 4 (3 pounds to a liter) averaged 44 million. Decrease in virulence was found to vary with the concentration of the meat infusion; the more dilute the infusion, the more rapid the loss. That meat infusion in the usual concentration lacks some essential for maintenance of virulence is suggested from the fact that a high concentration furnishes more suitable media than a lower concentration.

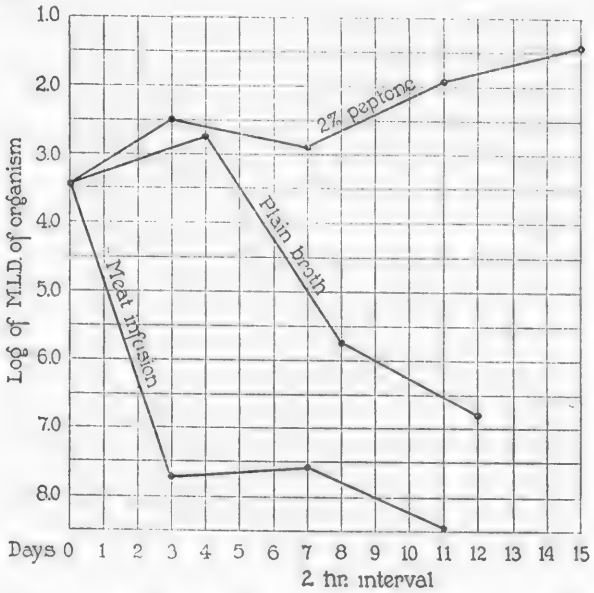


TEXT-FIG. 5. Effect upon virulence of variation in concentration of meat infusion.

Experiment 6. Effect of Peptone on Virulence of Pneumococci Transferred at 2 Hour Intervals.

This experiment was planned to test the effect of peptone on the virulence of pneumococci and to serve also as a check upon the results obtained in the previous experiments with regard to the influence of plain broth (beef infusion plus 1 per cent peptone) and of beef infusion.

The ingredients were uniform with these and the other conditions as nearly identical as possible. Since it had been found that pneumococci would grow to a slight extent on 2 per cent peptone with the addition of 0.5 per cent sodium chloride, these concentrations were used both in the test for the effect of peptone on virulence and for the control, plain broth, made by adding peptone to meat infusion (1 pound to a liter). All media were adjusted to pH = 7.4. The same strain of pneumococcus, somewhat avirulent, was used as in the previous experiments.



TEXT-FIG. 6. Effect of different ingredients of plain broth pH 7.4.

The results of this experiment (Text-fig. 6) are striking and to us surprising; the virulence of pneumococci grown on 2 per cent peptone during a period of 15 days, transferred every 2 hours, remained constant or even increased; on meat infusion, a rapid decrease occurred; while on plain broth, containing a mixture of ingredients favorable and unfavorable for maintenance of virulence, a decrease resulted and at a slower rate than occurred on meat infusion alone. Peptone neutralized to some extent the unfavorable action of meat

infusion, as did dextrose, described above. The outcome with peptone presents an interesting issue, for one could scarcely expect commercial peptone, a mixture of albumoses, peptones, and amino-acids, to be capable of furnishing conditions suitable at least for the preservation of the virulence of a fastidious organism like the pneumococcus. Although a reason cannot be assigned for the unexpected results with peptone, they may lead to simplification of present knowledge of the problem of virulence. The limited character of the studies with peptone, which, however, have since been substantiated, deter us from generalization. However, this much may be said. The virulence of the pneumococcus, as of other microorganisms, may be thought to be, perhaps, just as dependent upon definite chemical substances as the proliferation of the organism is upon certain food elements. One of the chief circumstances which determine virulence evidently is the presence or absence of certain chemical compounds. Life of the cells is possible with many different food elements, but virulence only with certain chemical substances, and these perhaps under special physicochemical conditions.

DISCUSSION.

Several inferences seem justified from the results presented. The H ion concentration of plain broth has a definite influence on the maintenance of virulence of pneumococci, but at no H ion concentration of this medium are conditions suitable for the permanent increase of virulence. Although an H ion concentration of the blood (pH = 7.4) was maintained in several experiments for a considerable length of time, the virulence of the pneumococci decreased, thus demonstrating that the H ion concentration alone of the blood does not insure the preservation of virulence even in young cells, much less fulfill conditions fit for the increase of this form of bacterial activity. Under the conditions of the experiments, regardless of the H ion concentration of plain broth or meat infusion, the virulence of the pneumococci was lost, the rate of loss depending upon the H ion concentration. In plain broth (Experiment 1) the more acid the media, the more rapid was the decrease; while in meat infusion, the reverse effect is to be noted. Seemingly, the OH ion is more destructive to virulence (Experiment 2) than the H ion. The

results reported in Paper II[1] show this fact in a definite fashion. When a virulent strain of pneumococcus was grown on milk adjusted to various H ion concentrations, that is pH = 5, 6, 7, 8, and 9, its virulence was maintained for over 100 transfers at a pH = 7 or below, while milk adjusted to pH = 8 or 9 caused in the same length of time a decrease in virulence, the more alkaline fluid having the more pronounced effect. To make a similar deduction from the experiments with plain broth is not possible for the reason that the virulence of pneumococci is lessened at all H ion concentrations studied. However, if the rate of decrease is used as the criterion, there is sufficient evidence to indicate that the OH ion in a constant medium possesses an action more antagonistic to virulence than the H ion. Other factors, without doubt, influence the virulence of pneumococci in ordinary media more than the H ion concentration.

No decision can be made at present as to what factors in plain broth media other than the H ion concentration play a rôle in determining whether a strain of pneumococcus grown thereon will be virulent or avirulent. However, the results obtained by a study of the effect on virulence of the two ingredients of plain broth, peptone and meat infusion, are worthy of comment. For it has been shown for one strain of pneumococcus that while the virulence of this organism decreased when it was grown on plain broth or on meat infusion—and with greater rapidity on the latter medium—in peptone solution virulence was not decreased, perhaps even increased. It is the meat infusion, then, not the peptone in ordinary plain broth, that has an unfavorable action on virulence. Furthermore, it has been shown that peptone neutralizes to a certain extent the unfavorable action of the meat infusion, as did dextrose. The question arises as to the mechanism of the action of the meat infusion, whether or not it is lacking in some substance necessary for the maintenance of virulence or contains some substance which has a specific action against virulence. Little can be said, now, on this point. If we take into consideration the effect of the concentration of meat infusion, which shows that the greater the concentration the less active this action, and also the fact that dextrose or peptone lessens it to some extent, we would be apt to conclude that the influence on virulence of meat infusion is due to a lack of some food element and

that it does not contain a substance antagonistic to virulence. On the other hand, if we consider that peptone alone furnishes the necessary conditions for the maintenance of virulence and that the addition of meat infusion destroys the favorable condition, a logical inference is that meat infusion contains a substance which diminishes the virulence of pneumococci.

SUMMARY.

1. A highly virulent strain of pneumococcus, Type I, Neufeld, when grown in an automatic transferring device at 2 hour intervals on different lots of plain broth adjusted respectively to pH = 6.5, 7, 7.5, or 7.8, lost its virulence at a rate in direct ratio to the H ion concentration of the media—the more acid the media, the more rapid the decrease.

2. Growth of a virulent pneumococcus upon plain broth of an H ion concentration changing gradually either to the acid or the alkaline side of neutrality was accompanied by an initial rise in virulence with a subsequent fall, the change being more rapid in the alkaline than in the acid medium. The organism under these conditions underwent an alteration in its behavior toward agglutinating sera; although still specific, its agglutinability became much greater than that of the original organism. This change was more pronounced in the alkaline than in the acid medium.

3. The virulence of a relatively virulent culture of a pneumococcus grown on plain broth underwent different alterations when the organism was transferred at intervals of 1, 2, 4, or 8 hours. With the 1 hour interval, there occurred seemingly an immediate decrease in virulence, while at intervals of 2, 4, and 8 hours, there occurred first a rise and then a fall, the rise being greatest with the 8 hour interval of transfer and least with the 2 hour.

4. Meat infusion adjusted to various H ion concentrations—pH = 7, 7.3, and 7.7—and made with different amounts of meat furnished conditions which caused a decrease in virulence, the unfavorable action varying in inverse proportion to the concentration of the infusion. Dextrose added to both plain broth and to meat infusion neutralized to a marked degree the unfavorable action of these media.

5. The growth of pneumococci on the different ingredients of plain broth, with plain broth made from the same supply as a control, revealed the fact that peptone in 2 per cent solution maintained and even increased the virulence of the strain of pneumococcus studied, while meat infusion caused the usual decrease. The rate of decrease with plain broth was slower than with meat infusion, showing that the 2 per cent peptone neutralized to some extent the unfavorable action of the infusion.

A REACTION PRODUCT IN INFECTIONS WITH TRYPANOSOMA LEWISI WHICH INHIBITS THE REPRODUCTION OF THE TRYPANOSOMES.

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INTRODUCTION.

The recent work of Taliaferro and Taliaferro (1, 2) has demonstrated that *Trypanosoma lewisi* exhibits a peculiar cycle of reproduction in its vertebrate host, the rat. According to this work, in both natural and experimental infections, when the trypanosomes first appear in the blood, they are always undergoing rapid reproduction. The rate of this reproduction, however, does not remain constant, but rapidly decreases so that eventually the trypanosomes do not show any evidences of multiplication or growth, but simply exist in the blood, sometimes in enormous numbers, until finally, after from a week to several months, they suddenly disappear from the blood and leave the rat immune to another infection. Such a gradual retardation and final inhibition of reproduction, as has been pointed out in the previous papers, is an evidence of some type of resistance by the host to the parasite. The object of the present paper is, primarily, to give experimental evidence of the formation, in the blood of the rat, of a reaction product which has this specific action on the trypanosomes, and supplementary to this, to present further data on the normal course of laboratory infections.

The present study is a continuation of our work on the resistance which a host develops against trypanosome infections. The term resistance is used, as in the earlier work, to denote collectively those factors, either active or passive, which operate adversely against the parasite and only that resistance will be considered which develops after the parasites have successfully invaded the host. The

sum total of such a resistance can be roughly measured by making daily determinations of the number of parasites for, if the parasites either remain constant or decrease in number, some type of resistance is operative. Any such change, however, may be brought about by one or both of two factors: (1) the rate of reproduction of the parasites may be retarded, or (2) the trypanosomes, after they are formed, may be destroyed. Thus:

Number of parasites per c.mm. of blood = Number produced by reproduction of parasites − Number destroyed in consequence of resistance.

To differentiate between these two factors a method has been developed of measuring the rate of reproduction, and hence, of getting the comparative number produced by reproduction of the parasites.

The only absolute measure of such a rate is probably the enumeration of all of the progeny which are formed, and is, obviously, impossible since, in parasitic infections, such as the present one, the host may kill large numbers of the parasites and thus mask any effect on the rate of reproduction. The measure devised is, therefore, indirect, but it is independent of the number of organisms killed as long as enough are left to furnish suitable samples for analysis. For a detailed description and demonstration of its validity, the reader is referred to Taliaferro and Taliaferro (1).

Essentially, the method consists in comparing the coefficient of variation, for some factor involving size, of samples of trypanosomes taken at stated intervals throughout the course of an infection. The rationale of this method is based on the obvious and well known fact that a sample taken on one hand from a population undergoing rapid reproduction, with the constant production of young forms and intermediate growth stages, will exhibit much greater variability in size than a sample taken on the other hand from a population in which there is little or no reproduction and in which all of the organisms are full grown adults.

Thus we have found, using the coefficient of variation for total length as the most reliable single constant in comparing variability, that, when the trypanosomes first appear in the blood, the coefficient of variation is about 30.0 per cent, which indicates a population of

trypanosomes undergoing reproduction at a maximum rate; that the coefficient of variation on succeeding days gradually drops, which indicates a cessation of reproductive activity; and that finally the coefficient of variation reaches a plateau at about 3.0 per cent, which represents the normal variation of non-reproducing adult forms.

To support these conclusions, direct examinations of stained blood smears show that dividing stages are extremely common on the 1st day after the trypanosomes appear in the blood, but gradually decrease until there are none at all during the later part of the infection.

Materials and Methods.

All of the work reported in this paper has been done on infections with *Trypanosoma lewisi* derived from the original pure line infection which was started in Rat 116 from a single trypanosome (Taliaferro (2)). The trypanosomes were always transferred from rat to rat either by intraperitoneal or intravenous injection and never by the invertebrate host. All intravenous injections were made with a 26 gauge Luer needle into one of the tail veins of a rat (generally 50 to 75 gm.) while under ether. After some practice, such injections proved to be fairly easy provided the rats were young and the skin of the tail was still soft.

The majority of the conclusions in the experiments which follow, are based on (1) the number of trypanosomes per c. mm. of blood; (2) the coefficient of variation for total length of the trypanosomes as a comparative measure of their rate of reproduction; and (3) as a check on (2), the direct examination of stained slides to determine whether trypanosomes were in the process of division. Each experimental rat, then, had to be examined daily, until the trypanosomes appeared in the blood, and then daily blood smears were made, dried, and stained with Wright's blood stain.

To obtain the number of trypanosomes per c.mm. of blood, the number of red cells per c.mm. of blood was ascertained by ordinary hemacytometer methods; the ratio between the number of trypanosomes and red cells found in a number of microscopic fields on a stained slide was ascertained; and with these data the number of trypanosomes per c.mm. of blood was calculated (Taliaferro and Taliaferro (1)).

To obtain the coefficient of variation (Taliaferro and Taliaferro (1)) the following steps were taken.

1. 50 trypanosomes were drawn by means of a camera lucida.
2. From these drawings the total length of each specimen was obtained in microns (total length includes free whip of flagellum).
3. The mean total length was obtained by dividing the sum of the measurements by the number of individuals (50).
4. The standard deviation was calculated by means of the formula

$$\sigma = \sqrt{\frac{\Sigma x^2}{n} - M^2}$$

in which x represents the magnitudes of the measurements, n the number of individuals, and M the mean.

5. Finally the coefficient of variation was derived by the formula $C.V. = \frac{100\,\sigma}{M}$ in which σ represents the standard deviation and M the mean.

The Normal Course of Laboratory Infections.

Before giving a detailed account of the experiments involved in the demonstration of the reaction product, it is essential to keep clearly in mind the course of ordinary infections.

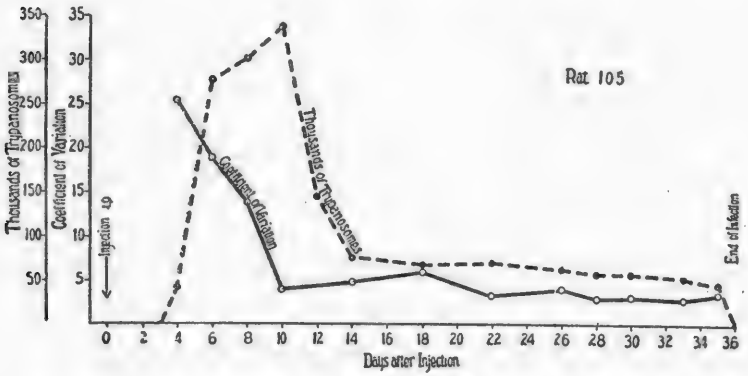
The life cycle of *Trypanosoma lewisi*, described previously by Taliaferro and Taliaferro (1), is well illustrated in Rat 105,[1] the findings in which are graphically shown in Text-fig. 1.[2] The trypanosomes first appeared in the blood (41,000 per c.mm. of blood) 4 days after an intraperitoneal injection of a dilute suspension of infected blood. They increased in numbers, although at a progressively lower rate, until the 10th day (338,000 per c.mm. of blood), when they suddenly decreased (76,000 per c.mm. on the 14th day), after which they remained relatively constant until the 36th day, when they disappeared altogether from the blood. The coefficient of variation on the other hand was extremely high (25.32 per cent) when the trypanosomes first appeared in the blood, but declined rapidly until the 10th day (3.95 per cent), after which it remained approximately at the level of the variability of non-reproducing adult trypanosomes; *viz.*, 3.0 per cent throughout the rest of the infection. This indicates that the rate of reproduction was very high when the organisms first appeared in the blood, that it rapidly decreased until reproduction was completely inhibited by the 10th day, and never resumed. Direct examination of the slides also showed that dividing forms were extremely

[1] The statistical constants in this infection were based on 100 trypanosomes each, instead of 50, the number used in the other infections in this paper.

[2] For original data see Taliaferro and Taliaferro (1).

numerous on the 1st day after the trypanosomes appeared in the blood, but that their number decreased until, after the 10th day, no dividing forms were encountered.

The results in this infection may be summed up in terms of the effects of the resistance developed by the rat, as follows: (1) As the result of an initial resistance, the rate of reproduction of the parasites was retarded more and more until it was finally inhibited altogether (by the 10th day). (2) A second effect of resistance developed between the 10th and 14th days when a large number of the parasites were destroyed. (This drop in numbers cannot be explained solely on the basis of the cessation of reproduction because, were this the only factor operative, the number of organisms would have remained constant.) (3) Finally,



TEXT-FIG. 1. Graph showing the coefficient of variation for total length (comparative measure of the rate of reproduction) and the number of trypanosomes per c.mm. of blood throughout the course of the infection with *Trypanosoma lewisi* in Rat 105.

a third effect of resistance developed by the 35th day when all of the trypanosomes left in the blood were destroyed. As far as can be ascertained at the present time these effects of resistance are due to different mechanisms.

In our previous work the conclusions reached in Rat 105 were checked in only one other infection (Rat 116). The normal course of events is so important, however, that detailed enumerative studies and statistical analyses were made of six other infections. As the chief interest of the present work is in that type of resistance which inhibits reproduction, it seemed necessary only to study that part of the infection which elapses before the trypanosomes reach the adult stage. Condensed protocols and the more important statistical constants and enumerative studies obtained in these infections are given in Table I, and the results for

TABLE I.

The Coefficient of Variation for Total Length and the Number of Trypanosomes per C.Mm. of Blood during the Course of Normal Laboratory Infections.

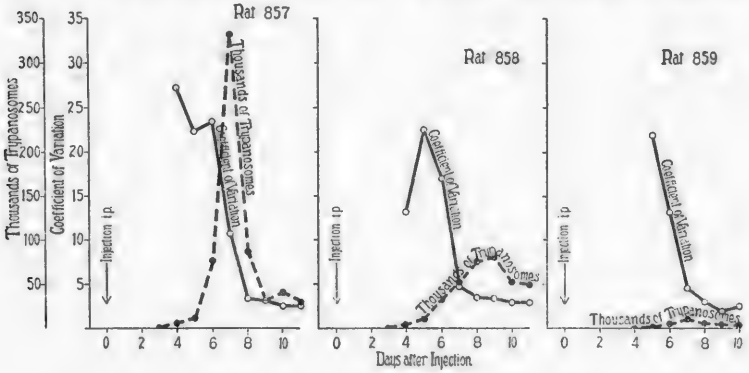
Length of time after injection.*†	Rat 857.		Rat 858.		Rat 859.		Rat 860.		Rat 861.		Rat 994.	
	Coefficient of variation.	No. of trypanosomes per c.mm. of blood.	Coefficient of variation.	No. of trypanosomes per c.mm. of blood.	Coefficient of variation.	No. of trypanosomes per c.mm. of blood.	Coefficient of variation.	No. of trypanosomes per c.mm. of blood.	Coefficient of variation.	No. of trypanosomes per c.mm. of blood.	Coefficient of variation.	No. of trypanosomes per c.mm. of blood.
days												
1												
2												
3											26.68	51,000
4	27.35	5,000	13.21	3,000			14.45	1,000	19.95	6,000	28.61	223,000
5	22.43	11,000	22.46	10,000	22.00	1,000	20.89	10,000	23.59	11,000	18.50	513,000
6	23.52	76,000	17.01	33,000	13.23	4,000	22.34	43,000	24.19	68,000	10.68	465,000
7	10.79	333,000	4.83	52,000	4.64	11,000	5.98	114,000	15.85	236,000	3.95	438,000
8	3.39	87,000	3.53	76,000	3.08	5,000	2.82		14.70	596,000	3.60	414,000
9	3.11	31,000	3.41	81,000	2.08	4,000	2.51	200,000	4.41	609,000	2.40	389,000
10	2.48	40,000	2.93	53,000	2.49	3,000	2.25	165,000	2.33	653,000		
11	2.51	29,000	2.85	49,000			2.38	130,000	2.46	559,000		

* All rats injected intraperitoneally with 1 cc. of a suspension of infected blood containing approximately 600,000 trypanosomes per cc. Rats 857 to 861 injected on September 23, 1922, and No. 994 on March 28, 1923.

† The first days of the infection (where there are no constants) represent the incubation period, during which there are no trypanosomes in the blood.

the three infections which are discussed in detail are graphically shown in Text-fig. 2.

Rat 857 is illustrative of the findings obtained in most infections (Rats 105, 116, etc.). After an incubation period of 4 days, when the trypanosomes first appeared in the blood, they exhibited a high coefficient of variation (27.3 per cent), which indicated that they were undergoing rapid reproduction. The coefficient of variation then dropped rapidly until by the 8th day it had reached the low value of 3.3 per cent, the normal variation of non-reproducing adult *Trypanosoma lewisi*. These facts were verified by direct microscopic examinations of the slides. Although no statistical computations were made after the 11th day, microscopic examinations of the slides indicated that, as in other infections, the organisms



TEXT-FIG. 2. Graphs showing the coefficient of variation for total length (comparative measure of the rate of reproduction) and the number of trypanosomes throughout the first parts of the infection with *Trypanosoma lewisi* in Rats 857, 858, and 859.

did not resume reproductive activity. The changes in the number of organisms per c.mm. of blood during this infection also check the results obtained in Rats 105 and 116. Between the 4th and 7th days, during active reproduction, the numbers rose from 5,000 to 333,000 per c.mm., but then rapidly decreased to 31,000 per c.mm. on the 9th day, after which they remained fairly constant until their final destruction.

It seems unnecessary to take up the other two rats in detail, but simply to call attention to the data in Table I and Text-fig. 2 and to point out variations.

As Rat 858 shows, the highest rate of reproduction is not always encountered when the trypanosomes first appear in the blood. In this case, the rate of reproduction, as indicated by the coefficient of variation, was considerably lower (13.2 per cent) on the 4th day, when the trypanosomes first appeared in the blood, than

on the 5th day (22.4 per cent) of the infection. In spite of this, however, the coefficient of variation (3.5 per cent) on the 8th day indicated that the trypanosomes had reached the adult stage, and hence, in this infection, as in Rat 857, there was no reproduction after the 8th day. The changes in the number of organisms during this infection, however, were markedly different from those of No. 857. True there was a recognizable initial rise (from 3,000 to 81,000 per c.mm. of blood) and fall (49,000 per c.mm. of blood), but the peak was comparatively low. This infection seems to indicate that the condition which produces the second effect of resistance, *i.e.* the partial destruction of the parasites after they are formed, must have been more or less operative during the entire multiplicative period.

The infection in Rat 859 represents the most extreme variation from the usual type and has been observed by us in only one case. The changes in the rate of reproduction, as indicated by the coefficient of variation, were similar to those in Rats 105, 116, etc. All reproduction ceased by about the 8th day. The number curve, however, is very aberrant. The slight peak in the numbers (from 1,000 to 11,000 per c.mm. of blood) on the 7th day was so small that it is doubtful whether the differences from day to day were very significant and it seems that the second effect of resistance (noted above) must have been operative to such an extent that most of the trypanosomes produced during the multiplicative period were killed. Furthermore, this infection is interesting because it illustrates the impossibility of measuring the rate of reproduction by changes in the number of trypanosomes in the blood throughout the infection. Such a criterion, in this infection in which the numbers scarcely increased at all, would indicate that there was little or no reproduction at any time, whereas a study of the coefficient of variation, as well as direct examination of the slides, reveals that reproductive activity was as intense as in any other normal infection (see No. 105, etc.). The constancy in the number curve was not due to a lack of reproduction but to a destruction of the trypanosomes after they were formed.

To summarize the work on normal infections, it may be said that the daily changes in the number of trypanosomes per c.mm. of blood vary considerably in different individuals, but that the changes in the rate of reproduction, as evidenced both by direct microscopic observations and by changes in the coefficient of variation, are extremely uniform. There is an initial high rate of reproduction when the organisms first appear in the blood followed by a rapid retardation and final inhibition of reproduction by about the 10th day of the infection.[3] These conclusions are also supported by the findings

[3] In our earlier work we stated that the multiplicative stage, which includes both the incubation period and the multiplicative period in the blood, lasted from 22 to 32 days. Mesnil (Mesnil, F., *Bull. Inst. Pasteur*, 1922, xx, 918) states that

in the various seed and experimental rats, as they may be termed, which are described in the next section.

Experimental Alterations in the Normal Course of Laboratory Infections.

If the inhibition of reproduction is due to the formation of a specific reaction product, then on the injection of a sufficient quantity of serum taken from the blood of a rat in which only adult trypanosomes exist, together with some adult trypanosomes, into a new host, the parasites, instead of repeating the normal course of the infection, should simply exist without manifesting any reproductive activity. The data which follow indicate that this is experimentally possible.

The experimental procedure was as follows: A rat, designated in our descriptions as the seed rat, was injected intraperitoneally with a dilute suspension of infected blood, and, after the trypanosomes appeared in the blood, daily determinations of the coefficient of variation for total length of the trypanosomes were made until all reproductive activity had ceased, as indicated by a 3 per cent coefficient of variation and the absence of any dividing forms. At this point, the rat was etherized, its thoracic cavity opened, and as much blood as possible drawn from the heart under aseptic conditions and centrifuged. After centrifugalization, the tube containing the blood was gently rotated so that the trypanosomes which were deposited just above the leucocytic cream were stirred into the serum, and the serum containing the trypanosomes and some white and a very few red corpuscles was decanted and recentrifuged. The trypanosome-free serum was removed and the trypanosomes in the sediment were washed three or four times in saline by separate centrifugalizations. At this point there was on the one hand trypanosome-free serum and on the other hand washed trypanosomes (with invariably a few white and red corpuscles) both of which came from the original seed rat. The final experimental procedure was to inject half of the

in his experience the period is much shorter. Strictly speaking we also found that "actual division probably ceases by the tenth day" and that "the slight decline [in the coefficient of variation] noticed from the tenth to the twenty-eighth day is probably the result of the growth of a few young forms still in the blood" (Taliaferro and Taliaferro (1)).

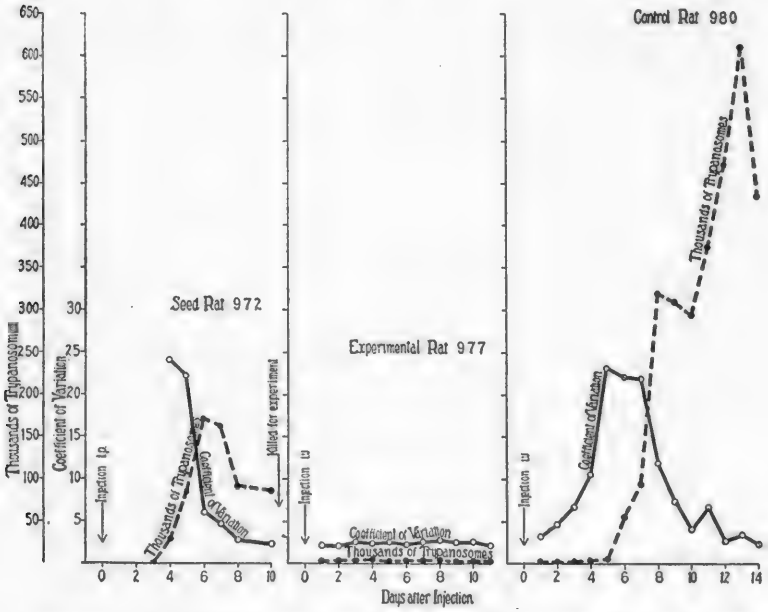
washed trypanosomes, mixed with a specified dose of serum, intravenously into a non-infected rat and the other half of the washed trypanosomes, mixed with a similar dose of normal serum or physiological saline, in the same manner into a similar rat. These two rats are designated in the later descriptions as the experimental and control rat, respectively. It is to be noted then that the experimental rat received a large intravenous dose of adult trypanosomes plus the serum in which they had been living but had been unable to reproduce, and that the control rat received an intravenous dose of approximately the same number of washed trypanosomes taken from the same source plus normal serum (or saline) in which presumably the trypanosomes could reproduce. If our contention is correct that inhibition of reproduction in normal infections is due to the formation of a specific reaction product in the serum then those adult trypanosomes which were injected into the experimental rat should not exhibit any reproductive activity, whereas those in the control rat should.

The procedure outlined above was finally adopted after a number of failures. Intravenous rather than intraperitoneal injection of the trypanosomes was used because it placed the trypanosomes directly in the blood where they could be under constant observation, whereas intraperitoneal injection occasioned an incubation period during which the organisms could not be located. Furthermore it was found necessary to inject large numbers of trypanosomes in order to find them in the experimental rats where they do not increase at all and control rats were given like doses so that they would be comparable to the experimental animals.

The following example is typical of the results obtained.

Seed Rat 972 was injected intraperitoneally, and, following the appearance of the organisms in the blood, after an incubation period of 4 days, daily determinations of the number of parasites per c.mm. of blood and the coefficient of variation were made. As can be seen by an examination of Text-fig. 3 and Table II, the results are closely similar to those obtained in Rat 105 and other ordinary infections. By the 10th day, the trypanosomes had ceased all reproductive activity and were in the adult stage. The experimental procedure outlined above was then carried out with Experimental Rat 977 and Control Rat 980 (compare results in Text-fig. 3 and Table II).

In the blood of Experimental Rat 977 (Text-fig. 3 and Table II), which received a dose of 2 cc. of serum per 100 gm. of body weight plus the adult trypanosomes, both from the seed rat, the trypanosomes were found very quickly after infection and for the first 11 days were sufficiently numerous to study. On the 12th and 13th days only an occasional organism was observed and none thereafter. Throughout the entire infection, none of the trypanosomes exhibited any indi-



TEXT-FIG. 3. Graphs showing the coefficient of variation for total length (comparative measure of the rate of reproduction) and the number of trypanosomes throughout the infections with *Trypanosoma lewisi* in Seed Rat 972, in Experimental Rat 977, and in Control Rat 980. Note that, in the experimental rat, which received a large number of trypanosomes plus 2 cc. of serum per 100 gm. of body weight, both from the seed rat, the normal cycle of reproduction was altered—there was a complete inhibition of reproduction, as evidenced by the coefficient of variation; whereas, in the control rat, which received a similar number of trypanosomes from the seed rat plus a similar dose of serum from a normal rat, the normal cycle of reproduction took place. The large number of trypanosomes in the control rat as compared with those in the seed rat is probably the result of the fact that the initial dose in the former contained at least a hundred times as many trypanosomes as that for the latter.

cations of reproduction, for the coefficient of variation remained between 2 and 3 per cent, and daily microscopic examinations of the slides failed to reveal any evidences of reproduction or growth. This conclusion is also corroborated by the fact that the numbers remained between 3,000 and 4,000 per c.mm. of blood during the entire infection; and, although, as we have already pointed out, changes in the numbers cannot be used indiscriminately to measure the rate of reproduction, in this case, an increased number of parasites would have indicated some reproduction, whereas a decreased or constant number would at least be what would be expected. It is, however, not conclusive evidence that reproduction

TABLE II.*

Experiment with Seed Rat 972, Showing Complete Inhibition of Reproduction in the Trypanosomes in the Experimental Rat.

Length of time after injection.	Seed Rat 972.		Experimental Rat 977.		Control Rat 980.	
	Coefficient of variation.	No. of trypanosomes per c.mm. of blood.	Coefficient of variation.	No. of trypanosomes per c.mm. of blood.	Coefficient of variation.	No. of trypanosomes per c.mm. of blood.
days						
1			2.09	2,000	3.34	2,000
2			2.08	3,000	4.72	1,000
3			2.61	3,000	6.75	2,000
4	24.17	29,000	2.38	4,000	10.67	3,000
5	22.31	85,000	2.53	2,000	23.20	5,000
6	6.05	171,000	2.28	2,000	22.21	55,000
7	4.63	163,000	2.53	3,000	21.93	96,000
8	2.70	91,000	2.70	3,000	12.00	320,000
9			2.45	1,000	7.41	310,000
10	2.22	86,000	2.49	1,000	4.19	295,000
11	Rat killed at this point for experiments with Rats 977 and 980.		2.12	1,000	6.77	374,000
12					2.73	472,000
13					3.55	613,000
14					2.38	435,000

*See Table IV for a condensed protocol of this experiment.

has not taken place because some destructive agent may have completely masked the effects of reproduction.

These results are in marked contrast to those in Control Rat 980 which received a dose of 2 cc. of normal serum per 100 gm. of body weight plus adult trypanosomes from Seed Rat 972. Here the organisms were likewise observable a very short time after they were injected into the blood. Careful study the first 14 days of the infection (Text-fig. 3 and Table II) revealed that, after 2 days, during which the trypanosomes remained in the adult stage, an intense period of reproduction, as is evidenced by the sudden increase in the coefficient of vari-

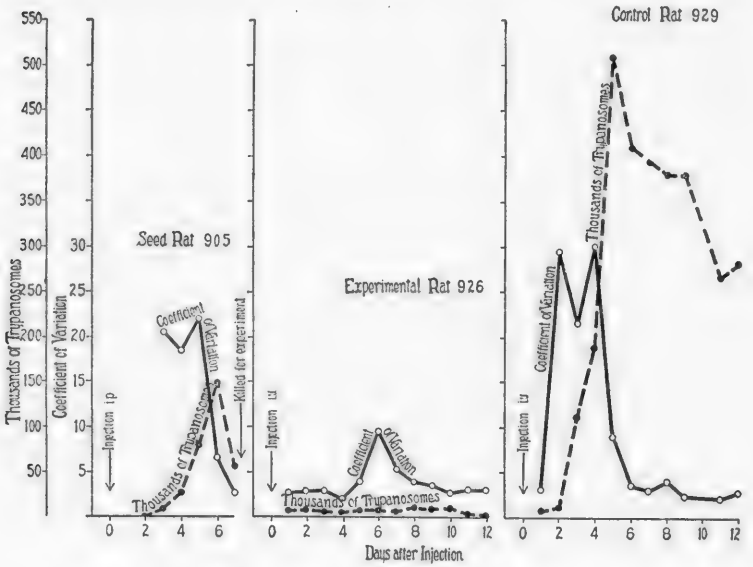
ation from 3.3 to 23.2 per cent, lasted until the 7th day, after which it rapidly subsided, so that by the 12th day only adult non-reproductive trypanosomes were present in the blood. The eventual inhibition of reproduction (by the 12th day) was of course due to the production of the reaction product in the rat itself just as in any normal infection. Microscopic investigation of the slides also indicated an intense period of reproduction between the 4th and 7th days, since there were a large number of dividing forms. Changes in the number of trypanosomes per c.mm. of blood also closely resembled those in a normal infection. The organisms increased to 613,000 per c.mm. of blood on the 13th day and thereafter rapidly decreased until they hit a more or less constant number. The fact that so many more trypanosomes were produced in the control rat than in the seed rat is probably due to the fact that the former received a much greater initial dose than the latter.

In other words, when the trypanosomes were injected into the blood stream of a fresh rat with the addition of the serum containing the reaction product developed in infected rats, they were unable to reproduce for at least 13 days (which was as long as any of the experimental infections lasted), whereas, when trypanosomes from the same source were injected with the addition of a like quantity of normal serum, they began reproduction after 2 days and underwent the ordinary cycle of reproductive activity.

Not all of the experiments show a complete inhibition of reproduction. By varying the dose of serum, different grades of inhibition were obtained. The following experiment is illustrative of those experiments in which almost but not quite complete inhibition took place (Text-fig. 4 and Table III).

Trypanosomes were injected into Seed Rat 905 intraperitoneally; first appeared in the blood on the 3rd day; went through the usual cycle of reproductive activity (Text-fig. 4 and Table III); showed a coefficient of variation of 2.5 per cent by the 7th day; and at this point were taken with a large amount of blood from the rat (No. 905). Half of them, plus the serum (1.6 cc. per 100 gm.) from the same rat, were then injected into Experimental Rat 926, and the other half, plus saline (1.6 cc. per 100 gm.), into Control Rat 929. The usual course of normal infections occurred in Control Rat 929 with the abrupt rise after 2 days and fall after 5 days in reproductive activity. In Experimental Rat 926, however, complete inhibition of reproduction lasted for 5 days. During the 6th and 7th days a slight indication of reproductive activity took place as evidenced by the rise in the coefficient of variation to 9.4 per cent. There can be no doubt that there was a partial inhibition because the trypanosomes showed no sign of reproductive activity until the

6th day, as against the 2nd day of the control; the peak of the coefficient of variation was only 9.4 per cent, as compared with 30.2 per cent; and microscopic examination of the slides on the 6th and 7th days showed that little, if any, actual reproduction took place, but rather that the rise in the coefficient of variation was chiefly due to the growth of the organisms preparatory to division. This conclusion is strongly supported by the fact that there was only a slight indication of any rise in the number of organisms per c.mm. of blood.



TEXT-FIG. 4. Graphs showing the coefficient of variation for total length (comparative measure of the rate of reproduction) and the number of trypanosomes throughout the infections with *Trypanosoma lewisi* in Seed Rat 905, in Experimental Rat 926, and in Control Rat 929. Note that, in the experimental rat, which received a large number of trypanosomes plus 1.6 cc. of serum per 100 gm. of body weight, both from the seed rat, the normal cycle of reproduction was altered—there was an incomplete inhibition of reproduction, as evidenced by the coefficient of variation; whereas, in the control rat, which received a similar number of trypanosomes from the seed rat plus a similar dose of physiological saline, the normal cycle of reproduction took place. The large number of trypanosomes in the control rat as compared with those in the seed rat is probably the result of the fact that the initial dose in the former contained at least a hundred times as many trypanosomes as that for the latter.

A number of experiments such as the two that have been given have been studied in detail and in each case daily determinations have been made of the number of trypanosomes per c.mm. and the coefficient of variation for total length. The results of these experiments are presented in Table IV. Although this table contains only a few experiments in which there was no inhibition in the experimental rats, a number of others which are not recorded were obtained in the first part of the investigations when a quantity of serum

TABLE III.*

Experiment with Seed Rat 905, Showing Partial Inhibition of Reproduction in the Trypanosomes in the Experimental Rat.

Length of time after injection.	Seed Rat 905.		Experimental Rat 926.		Control Rat 929.	
	Coefficient of variation.	No. of trypanosomes per c.mm. of blood.	Coefficient of variation.	No. of trypanosomes per c.mm. of blood.	Coefficient of variation.	No. of trypanosomes per c.mm. of blood.
days						
1			2.60	6,000	3.03	7,000
2			2.78	7,000	29.70	11,000
3	20.49	8,000	2.83	5,000	21.67	113,000
4	18.41	27,000	2.05	4,000	30.25	189,000
5	22.01	79,000	3.88	7,000	8.97	510,000
6	6.55	149,000	9.42	6,000	3.64	412,000
7	2.57	57,000	5.28	5,000	2.92	396,000
8	Rat killed at this point		3.85	10,000	4.17	381,000
9	for experiments with		3.52	8,000	2.43	381,000
10	Rats 926 and 929.		2.64	9,000		
11			2.91	3,000	2.19	267,000
12			3.07	1,000	2.75	282,000

* See Table IV for a condensed protocol of this experiment.

insufficient for inhibition was administered. Besides these data the formation of the reaction product is being repeatedly verified by work which is being carried on at the present time in this laboratory by Miss Frances A. Coventry.

The results in Table IV indicate that the titer of the serum for the reaction product increases during the course of the infection. Thus when the serum was obtained on the 5th day of the infection from the seed rats in the last experiment noted (eight seed rats used),

TABLE IV.

Reduced Protocols of Experiments Demonstrating the Inhibition of Reproduction in Trypanosoma lewisi by a Reaction Product in the Sera of Infected Rats.

Seed rat.			Experimental rat.			Control rat.			Inhibition of reproduction of trypanosomes.‡	
No. of rat.	Date of injection.*	Duration of infection when seed rat was killed.	No. of rat.	Weight.	Material injected intravenously.†	No. of rat.	Weight.	Material injected intravenously.†	Experimental rat.	Control rat.
		days		*gm.*			*gm.*			
972	Jan. 22, 1923	9	977	100	Trypanosomes + 2.0 cc. serum from Rat 972.	980	79	Trypanosomes + 2.0 cc. normal serum.§	+++	0
905	Nov. 13, 1922	9	926	63	Trypanosomes + 1.6 cc. serum from Rat 905.	929	63.5	Trypanosomes + 1.6 cc. physiological saline.	++	0
1010	Apr. 14, 1923	6	1012	68	Trypanosomes + 4.0 cc. serum from Rat 1010.	1013	100	Trypanosomes + 4.0 cc. normal serum.§	++	0
896	Nov. 7, 1922	8	899	60	Trypanosomes + 1.3 cc. serum from Rat 896.	910	72	Trypanosomes + 1.3 cc. physiological saline.	+++	0
914	" 16, 1922	11	940	93	Trypanosomes + 0.5 cc. serum from Rat 914.	948	69	Trypanosomes + 1.0 cc. normal serum.	+	0
			942	102	Trypanosomes + 0.20 cc. serum from Rat 914.				0	
966	Jan. 22, 1923	10	978	81	Trypanosomes + 2.0 cc. serum from Rat 966.	981	63	Trypanosomes + 2.00 cc. normal serum.	++	0
			979	57	Trypanosomes + 2.0 cc. serum from Rat 966.				+	

901	Nov. 13, 1922	5	922	61.5	Trypanosomes + 2.00 cc. serum from Rat 901.	924	56	Trypanosomes + 2.00 cc. physiological saline.	0	0
971	Jan. 22, 1923	10	974	70	Trypanosomes + 2.00 cc. serum from Rat 971.	976	96.5	Trypanosomes + 2.00 cc. normal serum.	+	0
872	Oct. 20, 1922	27	891	149	Trypanosomes + 0.7 cc. serum from Rat 872.				+++	
986	Feb. 27, 1923	13	989	89	Trypanosomes + 3.0 cc. serum from Rat 986.	990	80.5	Trypanosomes + 3.00 cc. normal serum.	++	0
‖	June 28, 1923	5	1034	107	Trypanosomes + 9.0 cc. serum from seed rats.				0	

* The infections in all seed rats were started by the intraperitoneal injection of 1 cc. of a very dilute suspension of infected blood.

† The trypanosomes injected into any given experimental rat and its respective control rat were always derived from the same source and were approximately equal in number. The doses of serum or physiological saline are not the actual quantity injected but the dose per 100 gm. of rat.

‡ +++ indicates the complete inhibition of reproduction, such as has been described in the experiment with Seed Rat 972 in which the trypanosomes in the experimental rat did not increase in number and never showed a coefficient of variation higher than about 3.0 per cent; ++ indicates almost complete inhibition of reproduction, such as has been described in the experiment with Seed Rat 905 in which the trypanosomes in the experimental rat increase very little, if any, in number, but the coefficient of variation showed a slight rise (10.0 per cent) about the 6th day of the infection; + indicates an experiment in which the trypanosomes in the experimental rat increased markedly in number and the coefficient of variation rose practically as high as in the control rats but reproduction did not begin until several days after the control; and 0 indicates no inhibition of reproduction, but simply the usual cycle of reproductive activity characteristic of ordinary infections which began about 2 days after the intravenous injection of the trypanosomes.

§ Control rats also run with physiological saline.

‖ The serum for this experiment was derived from eight seed rats.

while the trypanosomes were still undergoing reproduction, a dose of 9 cc. per 100 gm. was without effect on the reproduction of the trypanosomes. On the other hand, in Seed Rat 872, from which the serum was taken on the 27th day, complete inhibition of reproduction was obtained with a dose of only 0.7 cc. per 100 gm. This question of the increase in titer of the serum is being studied in this laboratory by Miss Coventry.

We have been unable to find any indication of the formation of any substance either similar to, or identical with the reaction product just described in *in vitro* cultures of *Trypanosoma lewisi*. The media used consisted of equal quantities of neutral beef broth and either rabbit or rat whole defibrinated blood. The following experiments are typical of the results obtained.

Nine culture tubes, prepared with rabbit blood, were inoculated with *Trypanosoma lewisi* on February 21, 1923. Examinations about every 3rd day revealed that organisms could be found, always in some cultures, irregularly in others; but that, whenever they were found, they were always in the process of reproduction, as indicated by numerous dividing forms and rosettes. This, in itself, indicates that no substance, operative in the cultures, is formed in the cultures which inhibits reproduction. Finally, on March 6, 1923, one of the cultures was centrifuged (no trypanosomes were found before centrifuging, but afterwards, a few, the majority of which were dividing, were found in the debris at the bottom), and the supernatant fluid injected intravenously into a rat (4 cc. per 100 gm.) together with a large number of washed "adult" trypanosomes. The resulting infection showed the normal cycle of reproductive activity with no inhibition of reproduction. Similar results have been obtained with other cultures made with rabbit blood and also with cultures made with rat blood.

DISCUSSION.

The primary object of the present paper is to present evidence of the formation of a reaction product in the serum of rats infected with *Trypanosoma lewisi*, which inhibits the reproduction of the trypanosomes. Although the effect on the parasite emphasizes a new method of resistance to an infection, the data at hand are insufficient for a decision as to whether the reaction product is different from those previously recognized. We shall summarize in this relation some work regarding its nature which is to be reported later.

In the introductory account of the resistance which a rat builds up to an infection with *Trypanosoma lewisi*, it was pointed out that there are three manifestations of resistance: (1) the retardation and final inhibition of reproduction in the trypanosomes occurring by about the 10th day; (2) a sudden drop in the number of organisms in the blood between the 9th and 15th days; and (3) the total disappearance of the organisms, which terminates the infection a week to several months after the first drop in numbers. The question arises: Is the reaction product with which we are dealing and which causes the first mentioned manifestation connected with the other two? The following observations suggest that it is not.

The third manifestation of those just mentioned is apparently due to a lytic agent which is operative in much smaller concentrations than the reaction product here under discussion. Scaling down the doses of serum containing the lysin has no effect on the rate of reproduction of trypanosomes in animals infected with it.

Up to the present time we have been unable to discover the mechanism of the initial drop in the number of trypanosomes. We have failed to link this partial disappearance of the organisms with (*a*) a migration to the tissues other than the blood, (*b*) phagocytosis, and (*c*) lysis. The possibility that it may be due to a fixed tissue phagocytosis which for some reason is discontinued before all of the organisms are destroyed has not been adequately tested. It is perhaps significant that in all infections allowed to run their course, complete or almost complete inhibition of reproduction occurs prior to the drop in numbers, whence it follows that enough of the reaction product must be present to inhibit reproduction before there is any marked destruction of the trypanosomes. The fact that it has been impossible to demonstrate the presence of the reaction product in the serum before the drop in numbers occurred may be due to the low titer of the serum, which makes it impossible to obtain large enough quantities. The possibility remains that the second manifestation of resistance is effected only after the concentration of the reaction product has reached a certain point. It is known, however, that the titer of the serum for the reaction product increases with the age of the infection, and, consequently, it should be expected that the destruction of the trypanosomes would continue as the titer increases. This has not been found to be true. Thus, all of the avail-

able evidence indicates that the reaction product which inhibits reproduction acts neither to kill the trypanosomes nor to affect their vitality.

In conclusion, it may be noted that while not lethal in its activities the present reaction product may play a significant part in the immunity of recovered rats to *Trypanosoma lewisi*. Preliminary investigations indicate that the lysin which terminates the infection persists in circulation only a short time, whereas the reaction product is present in the serum of recovered rats for many months. With this reaction product in the serum, a rat is, to all intents and purposes, as resistant to an infection as one which possesses a lysin, because the few trypanosomes which might enter the body are incapable of multiplying and setting up an infection. The formation of the reaction product early in an infection would explain why superinfections do not occur.

SUMMARY.

1. In infections with *Trypanosoma lewisi* in the rat there are three manifestations of resistance against the parasite: (1) the retardation and final inhibition of reproduction of the trypanosomes by about the 10th day; (2) a sudden drop in the number of parasites in the blood between the 9th and 15th days; and (3) the total disappearance of the parasites, which terminates the infection and occurs from a week to several months after the first drop in numbers.

2. The first manifestation of resistance, *viz.* the retardation and final inhibition of reproduction, is the result of the formation of a reaction product in infected rats which inhibits reproduction of the trypanosomes but does not kill them.

3. No product similar to, or identical with this reaction product can be demonstrated in *in vitro* cultures of *Trypanosoma lewisi*.

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